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On Body Size and Tissue Respiration

MARTIN DAVIES
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It is well known that the relation between the basal metabolic rate (B.M.R.) of homoiotherms, and their body weight, is of the form $H = \alpha\ W^{-\beta}$ (Brody, '45, p. 368; and Kleiber, '47). According to Brody, if H is expressed in kilocalories per hour, and W in kilograms, $\alpha = 2.92$ and $\beta = 0.73$. There has been much discussion of the contributions of the principal tissues to the B.M.R. of animals of different body weight, particularly with regard to the means by which the contributions of these tissues to the B.M.R. are determined by the rates of metabolic processes occurring in their cells. Unfortunately there is little direct evidence of the metabolic rates of homologous tissues of different animals measured *in vivo*, so that the basis of these discussions has mainly been the Q_{O_2} of isolated tissues. The conclusions which have been reached for other tissues, rest on the assumption that the Q_{O_2} of the excised tissue accurately represents the metabolic rate of the tissue in the intact animal under basal conditions, that is to say, using conventional Q_{O_2} units, that Q_{O_2} *in vivo* equals Q_{O_2} *in vitro* for any tissue. It is generally admitted that this assumption is not valid for skeletal muscle, since neither the integrity of the tissue, nor its basal tone can be maintained when using normal manometric procedures. The contribution of skeletal muscle cannot therefore be estimated from the very varied Q_{O_2} values which have been found *in vitro*. To test rigorously for other tissues the assumption that Q_{O_2} *in vivo* equals Q_{O_2} *in vitro*, it would be necessary to measure the oxygen uptake of each organ under basal conditions *in vivo* by the method of A.V. difference, and afterwards to kill the animal and measure the Q_{O_2} of representative slices from each organ, in standard media such as those of Krebs.

Although it is understandable that this test has never been made, it is regrettable that the assumption should seldom, if ever, have been clearly stated. An attempt is made here to test the assumption that Q_{O_2} *in vivo* equals Q_{O_2} *in vitro* indirectly, using data from the literature. It is concluded that when Q_{O_2} is determined *in vitro*, using a standard medium appropriate for the tissue, it is for most tissues an overestimate of Q_{O_2} *in vivo*. In reaching this conclusion it has been necessary to raise some other questions, which have entailed reviewing and to some extent reinterpreting the evidence on the relation of tissue metabolism to body size. The following is a general outline of the whole argument, which is pursued in detail below.

A relation between Q_{O_2} measured *in vitro* and the body weight W is established for the principal non-muscular tissues of various animals other than man. After showing that it is satisfied by the Q_{O_2} for human brain measured *in vitro*, the relation is used to find by interpolation values of Q_{O_2} *in vitro* for other human tissues. Values of Q_{O_2} for human tissues, estimated *in vivo* from experiments on the regional circulation of man at rest, are corrected to basal conditions and compared with the corresponding value of Q_{O_2} *in vitro* for man.

The relation between Q_{O_2} in vitro, and body weight

Krebs ('50) determined the Q_{O_2} of brain (cortex), liver, lung, kidney, and spleen from 9 species, but did not make a quantitative analysis of the relation between Q_{O_2} and body weight. He devised standard media containing intermediates of the tri-carboxylic acid cycle and found that in his calcium free medium, medium II, brain and liver gave high results. For these tissues medium III, containing calcium and bicarbonate, was preferred. Figures 1, 2 and 3 show Krebs' results for kidney, lung and spleen in medium II, in the form of graphs of log Q_{O_2} against log W. His results for brain and liver in medium III are plotted in figures 4 and 5, and in addition the relations for medium II are shown as dotted lines. In fitting these straight lines by the method of least squares, a point has been neglected in each of figures 1, 2 and 3. These points are clearly distinguished from the rest in the graphs. Since log Q_{O_2} is proportional to log W, $Q_{O_2} = kW^{-p}$, where $-$ p is the slope of the graph.

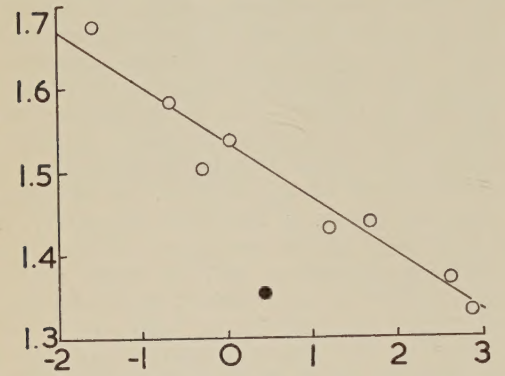

Fig. 1 Kidney, medium II. Ordinate: log Q_{O_2}. Abscissa: log W. ●, neglected in the least squares calculation; $k = 1.53$, $p = 0.068$.

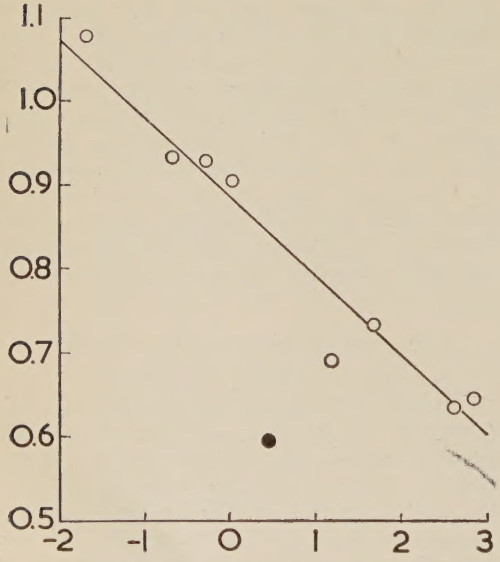

Fig. 2 Lung, medium II. Ordinate: log Q_{O_2}. Abscissa: log W. ●, neglected in the least squares calculation; $k = 0.89$, $p = 0.098$.

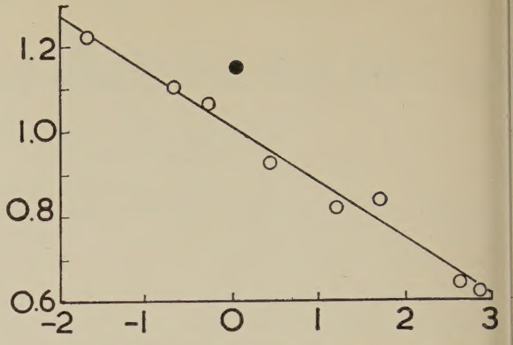

Fig. 3 Spleen, medium II. Ordinate: log Q_{O_2}. Abscissa: log W. ●, neglected in the least squares calculation; $k = 1.01$, $p = 0.133$.

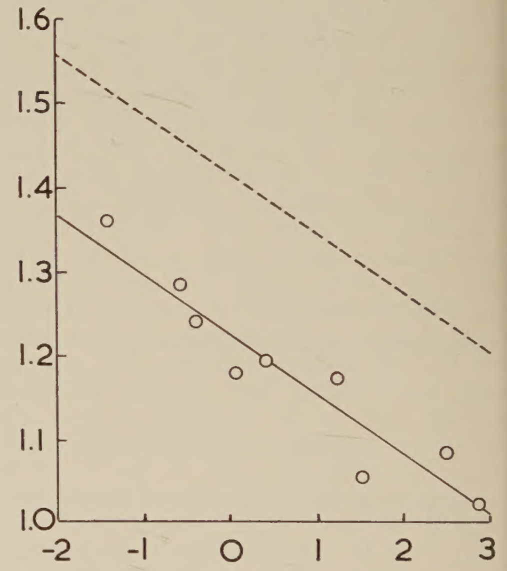

Fig. 4 Brain. Ordinate: log Q_{O_2}. Abscissa: log W. Medium III, solid line and points; $k = 1.23$; $p = 0.072$. Medium II, broken line; $k = 1.41$, $p = 0.068$.

Elliott ('48) has measured the oxygen consumption of slices of cerebral cortex in two media without tri-carboxylic acid cycle intermediates, one with calcium and bicarbonate, and the other calcium free. Figure 6 is redrawn from his work. Elliott did not determine the dry weights of all his slices, but gave the oxygen consumption in ml O_2/gm wet weight/hour; however he determined the percentage water of some samples as 80%, and this figure

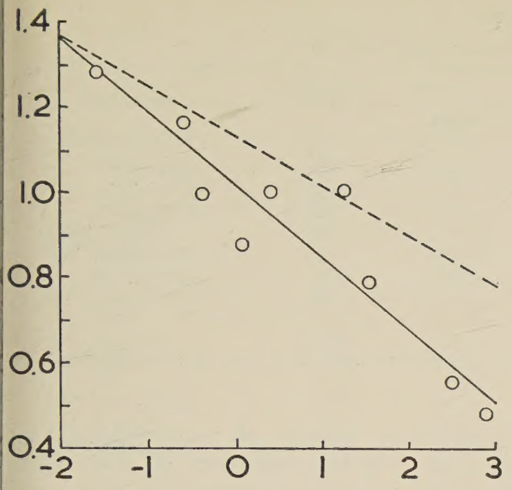

Fig. 5 Liver. Ordinate: log Q_{O_2}. Abscissa: log W. Medium III, solid line and points; k = 1.02, p = 0.172. Medium II, broken line; k = 1.14, p = 0.115.

has been used to convert his results into Q_{O_2} units, and so to obtain k in the equation $Q_{O_2} = kW^{-p}$. It can be seen that p, which is independent of the units in which metabolic rate is expressed, has the same value (0.1) for both media, but is not in good agreement with the values from Krebs' work, where p = 0.068 for medium II, and 0.072 in medium III. Both workers found that the rate of respiration was higher in the calcium-free medium.

Elliott found that there were considerable variations between normal individuals of the same species, and between different sites on the same brain. The value for human brain is the mean of experiments on 10 samples taken by Penfield from epileptogenic tissue (Elliott and Penfield, '48). There were large differences between the rates of individual samples, but since there was no correlation of metabolic rate with the site of epileptogenic focus, Elliott and Penfield concluded that the neurological disorder was not correlated with the variations of Q_{O_2}, and that the mean oxygen uptake of the samples was a representative value for normal cerebral cortex.

This was confirmed by Pappius and Elliott ('54), who found no significant difference between the Q_{O_2} of normal and epileptogenic cortical tissue, measured in Krebs' medium II. They also measured the Q_{O_2} of rat, cat and beef cortex in this medium, and their results were consistent with Elliott's plot in the calcium free me-

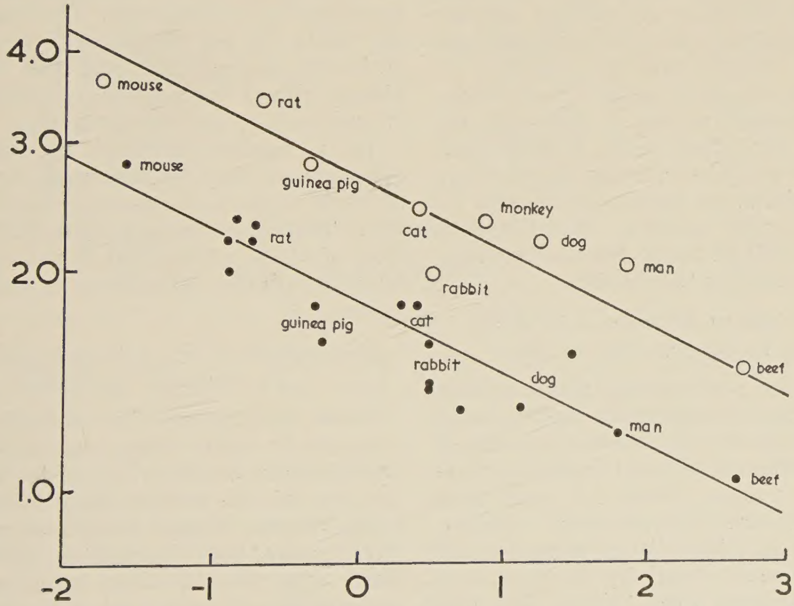

Fig. 6 Brain. Redrawn from Elliott ('48). Ordinate: oxygen uptake, in ml/gm/hour (logarithmic scale). Abscissa: log W. ○, in calcium free phosphate buffered medium, k = 1.13, p = 0.1; ●, in complete bicarbonate buffered medium, k = 0.96, p = 0.1.

TABLE 1

The Q_{O_2} of human brain, in vivo and in vitro

Method	Q_{O_2}		H_2O	Reference
	Whole brain	Cortex	%	
Krebs' medium III (interpolated)	$(9.9)^2$	12.4		Krebs, '50
In vivo	10.8		80^1	Kety, '57
In vivo	9.3		80^1	Bard and Bazett, '56
(from table 4)	7.45		75^1	
Glucose/Ca, electrical activation	8.4	(10.5)	80	McIlwain, '59
Glucose/Ca	(4.8)	6.0	80	Elliott, '48
Glucose/Ca	4.48	(5.6)	80	McIlwain, '59

[1] Assumed.

[2] Q_{O_2} values in parentheses were calculated on the assumption that Q_{O_2} whole brain = 0.8 Q_{O_2} cortex. (Elliott, '55).

dium, that is, significantly lower than Krebs' results in the same medium.

The Q_{O_2} of human brain in vitro

Brain is the only major human tissue of which the Q_{O_2} has been measured *in vitro*. Values of Q_{O_2} *in vitro* are given in table 1, together with the Q_{O_2} for a 70-kg man interpolated from figure 4 (medium III), and values of Q_{O_2} *in vivo*. The figures in brackets for whole brain, are calculated as 80% of the corresponding ones for cortex. The basis of this conversion factor is given by Elliott ('55).

McIlwain ('59) has developed a method of activation of slices by means of the application of electrical pulses, which has the effect of roughly doubling the Q_{O_2} of the slices. Brain, even under basal conditions, is electrically active, so although the value of 9.9 for whole brain from Krebs' data appears to agree with the Q_{O_2} *in vivo*, it should perhaps be multiplied by two to simulate electrical activity. Bearing this in mind, 9.9 will be taken for the moment as the best value for Q_{O_2} *in vitro*.

Basal metabolism of man, calculated from Q_{O_2} measurements in vitro

In table 2 the non-muscular metabolism of an ideal man weighing 70 kg has been estimated from the Q_{O_2} values of isolated mammalian tissues selected from the literature. Two values, those for skin and erythrocytes, are for human tissues. Values for brain, liver, lungs, kidneys and spleen have been found by interpolation from the graphs of Krebs' data. The rest are from experiments on various species, and are mostly not corrected for the different body weight of man. Details of sources, and the selection of data from them, are given in an appendix. The basal metabolic oxygen consumption of a 70-kg man with a respiratory quotient of 0.85, is taken as 13.8 liters per hour. The estimated non-muscular metabolism is therefore 126% of this B.M.R. There is not enough data available for the calculation of rational corrections to the body weight of man based on $Q_{O_2} = kW^{-p}$; arbitrary corrections can, however, be applied to the most doubtful Q_{O_2} figures in order to reduce the estimate to 106% of the B.M.R. Table 3 shows the details for those tissues affected. The value taken for brain is for slices of human brain activated electrically, and the value for kidney allows for medullary tissue, which is assumed to have a lower Q_{O_2}.

In a further attempt to reduce the estimate, it may be assumed that 12.5% of each organ is supporting tissue of zero Q_{O_2}. This gives a non-muscular metabolism of 110% of the B.M.R. in table 2, and 93% when the corrections in table 3 are made.

Metabolism of man from experiments on regional circulation

Some Q_{O_2} values in table 2 may be compared with those calculated from experiments on circulation in man. Estimates of the contribution of the principal tissues to the total resting metabolism have been compiled (Bazett, '49; Bard and Bazett, '56) from studies of regional blood flow, and A.V. differences. The organ weights and tissue oxygen uptakes given in table 4 for a 63-kg man, are

TABLE 2

Metabolism of man from Q_{O_2} in vitro

	Tissue	% body wt.	Wet wt.	H_2O	Dry wt.	Q_{O_2}	l O_2/hr.
			kg	*%*	*kg*		
1	Brain and	2.07	1.450	75	0.363	9.9	3.600
1A	spinal cord	0.64	0.450	75	0.112	9.9	1.110
2	Liver	2.50	1.750	71	0.508	5.07[1]	2.576
3	Kidneys	0.44	0.310	70	0.093	25.6[1]	2.381
4	Lungs	1.86	1.300	70	0.390	5.15	2.009
5	Intestine and stomach	2.00	1.400	77	0.322	5.0	1.610
6	Bone marrow	2.9	1.530	70	0.459	3.3	1.514
7	Skin	7.00	4.900	58	2.058	0.6	1.235[2]
8	Adipose	12.00	8.400	23	6.468	0.07	0.453
9	Spleen	0.25	0.175	70	0.053	5.83[1]	0.309
10	Testes	0.11	0.080	70	0.024	11	0.264
11	Pancreas	0.15	0.105	70	0.032	5.2	0.166
12	Thyroid	0.04	0.025	70	0.008	9.0	0.072
13	Adrenals	0.02	0.015	70	0.005	10	0.050
14	Thymus	0.03	0.020	70	0.006	5.5	0.033
15	Blood cells	3.14	2.200	72	0.616	0.017[2]	0.011
16	Heart	0.50	0.350	70	0.105	Total	17.395
17	Muscle	45.89	32.120	72	8.994		
18	Plasma	3.89	2.700	94	0.162		
19	Skeleton (less marrow)	15.31	10.720	22	8.362		
	Whole body	100	70	57.73	29.590		

[1] Krebs.

[2] Human.

TABLE 3

Corrections to table 2

	Tissue	Correction	l O_2/hr.
1	Brain	$Q_{O_2} = 8.4$	3.049
1A	Spinal cord	$Q_{O_2} = 8.4$	0.941
3	Kidneys	$Q_{O_2} = 21$	1.935
7	Skin	$\frac{3}{4}$ Q_{O_2}	1.355
5	Intestine and stomach	$\frac{1}{2}$ Q_{O_2}	0.805
8	Adipose	$\frac{1}{2}$ Q_{O_2}	0.227
10	Testes	$\frac{1}{2}$ Q_{O_2}	0.132
11	Pancreas	$\frac{1}{2}$ Q_{O_2}	0.083
13	Adrenals	$\frac{1}{2}$ Q_{O_2}	0.025
14	Thymus	$\frac{1}{2}$ Q_{O_2}	0.017
	Total		8.587
	Total from table 3 for these tissues		11.290
	Difference		2.703
	Non-muscular metabolism from table 2		17.395
	Non-muscular metabolism, after correction		14.692

based on those of Bazett and Bard. The percentages of tissue water are the same as those used in table 2, and the whole body has been adjusted to the same percentage of water. The ratio r is defined as the ratio of the Q_{O_2} given in table 2 or table 3 to that for the same tissue in table 4. The absolute Q_{O_2} will be incorrect if the tissue water has been wrongly estimated, but r will not be affected since the percentages of water are the same in both tables.

The basal metabolism of a 63-kg man is taken as 13 l O_2 per hour, which is 83% of the resting rate given in table 4. Basal Q_{O_2} values have been estimated from the resting values on three alternative assumptions.

1. That the muscle metabolism alone is reduced to bring the total oxygen uptake down to the basal value. The difference of 2.66 l O_2 between the resting and basal metabolism is divided in such proportions that the metabolism of the heart and re-

TABLE 4

Metabolism of man from experiments on regional circulation

Tissue	% of body wt.	Weight	Water	Dry wt.	l O_2 per hr.	Resting Q_{O_2}	Basal Q_{O_2} found under assumptions in text			Ratio r	
							1	2	3	Table 2	Table 3
		kg	%	*kg*							
Hepatic portal	4.13	2.6	71	0.75	3.06	4.08	4.08	3.39	3.87	1.31	1.31
Brain	2.22	1.4	75	0.35	2.76	7.89	7.89	6.52	7.45	1.33	1.13
Kidneys	0.48	0.3	70	0.09	1.08	12.0	12.0	9.96	11.4	2.24	1.84
Skin	5.72	3.6	58	1.51	0.72	0.48	0.48	0.40	0.46	1.30 (1.50)[2]	0.98 (1.13)[2]
Heart	0.48	0.3	70	0.09	1.74	19.3	16.0	16.0	18.3		
Respiratory and	0.64	0.4	72	0.11	0.66	6.00	5.0	5.0	5.72		
skeletal muscle	49.20	31.0	72	8.68	3.00	0.35	0.086	0.29	0.33		
Residual	37.13	23.4	36[1]	15.05[1]	2.64	0.18	0.18	0.15	0.17	2.12	1.76
Totals	100	63		26.63	15.66						

[1] Chosen so as to bring % H_2O of body to 57.73%.

[2] Basal Q_{O_2} under assumption 2.

spiratory muscles are decreased by th same percentage as the total metabolism and skeletal muscle accounts for the res of the decrease, being reduced to 25% o its original value.

2. That all the Q_{O_2} values are reduce to 83% of their rest values.

3. That the values found under the sec ond assumption are corrected by allowin for an arbitrarily chosen quantity, 12.5% of each organ, of inactive supporting tis sue of zero metabolic rate, which woul be dissected out by an experimenter de termining the Q_{O_2} *in vitro*.

The Q_{O_2} of residual tissues in table 2 is found to be 0.36, from the sums o oxygen uptake and dry weight for tissues 1a, 4, 7, 10 to 15, 18 and 19. A correcte value, 0.30, for the Q_{O_2} of these tissues is obtained in the same way, but substi tuting the Q_{O_2} in table 3 for tissues 1a 7, 8, 10, 11, 13 and 14.

The ratio r is given for both tables 2 and 3. The Q_{O_2} used from table 4 was the basal value found under assumption 3. Since it would not be possible to dissect inactive supporting tissue out of skin, the values of r under assumption 2 are also given, and are to be preferred. The only values of r less than 1.25 are those for brain (taking the Q_{O_2} of electrically activated human tissue calculated from McIlwain's figures) and for skin.

Regional metabolism of animals other than man

Table 5 shows Q_{O_2} values calculated from circulation and perfusion experiments on animals other than man, together with values from Krebs' data for comparison. The Q_{O_2} of perfused lung is very much lower than that of slices of lung, and altogether more values of Q_{O_2} determined *in vivo* are low than high when compared with the corresponding value interpolated from the graphs.

Comparison of regional metabolism of rat, man and cow

Table 6 is a comparison of the contributions of the tissues studied by Krebs, to the basal metabolism of three animals of very different size. The Q_{O_2} values for rat and cow are Krebs' experimental figures, supplemented by a doubled value fo

TABLE 5

Regional metabolism of animals other than man, in vivo

No.	Organ	Animal	Method	No. of expts.	Body wt.[1]	O_2 consumption (authors' units)	Organ wt. (assumed)	% Solids (assumed)	Q_{O_2}	Q_{O_2} *in vitro*[2]	*Literature reference*
					kg	*ml/100 gm/min. unless stated*	*gm*				
1	Liver	Dog	Unanesthetized. Double balloon cannula.	7	16	4.5		29	9.3	6.5	Blalock and Mason, '36
2	Liver	Dog	Unanesthetized. Double balloon cannula.	2	10, 11	32.1 ml/min.	420[3]	29	17.7	7.0	Levy and Blalock, '37
3	Liver	Dog	Anesthetized. B.S.P.	41	16	38 ± 15 ml/min. 7.9	480 (authors')	29	16.4	6.5	Werner et al., '52
4	Liver	Cat	Simple flowmeter								Barcroft and Shore, '12–'13
			Fasted	7	(2.75)	1.1		29	2.3	8.8	
			Fed	5	(2.75)	3.5		29	7.3	8.8	
5	Lung	Dog	Isolated lung, perfused from donor dog.	28	10	$Q_{O_2} = 0.071$			0.071	6.2	Bostroem and Lochner, '55
6	Kidneys	Dog	Unanesthetized	2	10, 11	4.6 ml/min.	70[3]	30	12.2	29	Levy and Blalock, '37
7	Kidneys	Dog	Various		(10.5)	10.7		30	21.4	29	Green, '50
8	Brain	Cat	Perfusion. Conscious?	6	3	4.95–4.01		20	12–15	15.6 (12.5)	Geiger and Magnes, '47
9	Brain	Dog	Anesthetized. Bubble flowmeter.	4	8	2.8		20	8.4	14.5 (11.6)	Noell and Schneider, '48
10	Brain	Monkey	Anesthetized. Bubble flowmeter.	11	2.8–4.6	3.7		20	11.1	15.3 (12.2)	Schmidt et al., '45
11	Brain	Rat	Anesthetized.	10	0.150	0.056 ml/min.	0.9 (0.6% of W)	20	18.7	19.5 (15.6)	Bergen et al., '53

[1] Figures in parentheses assumed.
[2] Figures in parentheses are 0.8 × Q_{O_2} brain cortex, to simulate the Q_{O_2} of whole brain (Elliott, '55).
[3] Brody, '45, p. 642.

TABLE 6

Comparison of regional metabolism of rat, man and cow

Tissue	Rat, 0.21 kg			Man, 70 kg			Cow, 420 kg		
	Q_{O_2}	% Body wt.	ml. O_2 per hr.	Q_{O_2}	% Body wt.	Liters per hr.	Q_{O_2}	% Body wt.	Liters per hr.
Brain	15.4[1]	0.6	4.91	9.9	2.07	3.60	9.7[1]	0.083	0.85
electrically activated	30.8[2]		9.82	8.4		3.05	19.4[2]		1.70
Liver	14.6	3.8	33.8	5.07	2.50	2.59	3.6	1.1	4.79
Lungs	8.6	0.70	3.78	5.15	1.86	2.01	4.3	0.6	3.23
Kidneys	38.2	0.78	18.72	25.6	0.44	2.38	23.5	0.2	5.88
Spleen	12.7	0.20	1.65	5.83	0.25	0.31	4.4	0.13	0.70
Total			62.93			10.89			15.45
B.M.R. R.Q. = 0.85			178[3]			13.8			71.2[3]
Total as % of B.M.R.			35%			78.8%			22%
Total, with value for activated brain			73.84			10.34			16.30

[1] Q_{O_2} whole brain = 0.8 Q_{O_2} cortex (Elliott, '55).

[2] Assumed.

[3] From the B.M.R. per unit wt. quoted by Krebs, '50.

brain, to simulate the results of electrical activation. The figures for man come from table 2. The organ weights for rat and cow are typical values consistent with the literature (Freudenberger, '32; Swett et al., '37).

The figures for the rat may be compared with those of Field, Belding and Martin ('39) who made determinations of Q_{O_2} in Ringer-glucose for all the tissues of the rat. The summed respiration accounted for 65.8% of the B.M.R. For the 5 tissues of table 6, the sum was 17% of the B.M.R. Even if all the Q_{O_2} values are multiplied by 1.52, to make the respiration accounted for equal to the B.M.R., and so allow for technical imperfections in the measurements, the sum for the 5 tissues is only 26% of the B.M.R. The corresponding figure in table 6 is 36%.

DISCUSSION

Empirical equations of the form $Y = uX^{-v}$ are frequently used in biology; they are useful expressions of trends, although they can seldom be used to predict Y accurately for a known value of X.

Brody has shown that for a wide range of mammals the weight of an organ, w, is related to the body weight W, by the equation

$$w = a\,W^{-m}. \tag{1}$$

Each organ has a specific value of a anc m (Brody, '45, p. 591).

$$W = \Sigma w = \Sigma a W^{-m}. \tag{2}$$

Brody gives m values for a number of tissues: for heart, lung and blood, m is approximately one, and for all other viscera it is less than one. By equation 2, m must therefore be greater than one for some other tissues; on the basis of dimensional analysis (Thompson, '42) the skeleton and supporting tissues must certainly be among these, though muscle need not be.

In order to find Q_{O_2} as a function of W, it is next assumed that

$$h = dW^{-n}, \tag{3}$$

where h is the heat production of the organ or tissue in kilocalories per hour. The Q_{O_2} *in vivo* is related to h by the equation

$$Q_{O_2}\ in\ vivo = \frac{h}{csw}. \tag{4}$$

where c is the calorie equivalent of a liter of oxygen for the tissue under consideration, and s is the solid fraction, or dry weight per unit wet weight of the tissue; c and s are both assumed to be constants for the tissue over the whole range of W. For the sake of mathematical convenience it is now assumed that Q_{O_2} *in vitro* is a stant proportion of Q_{O_2} *in vivo* for all that is:

$$Q_{O_2} \textit{ in vitro} = r\,Q_{O_2} \textit{ in vivo}. \qquad (5)$$

m equations 3, 4 and 5

$$Q_{O_2} \textit{ in vitro} = \frac{rdW^{-n}}{csw}$$

d eliminating w by means of equation 1

$$Q_{O_2} \textit{ in vitro} = \frac{rd}{acs}(W)^{m-n}. \qquad (6)$$

uation 6 might be modified by supposing t either c or s, or both, were related to by equations of the form $Y = uX^{-v}$, but s would not alter the form of the result. ere is little comparative data on tissue ter, and none on calorie equivalents; wever, all cells require between 60 and % of water for life, and the substances tabolized are qualitatively the same over e whole range of W, so that if c and s re functions of W, the values of the nstants in the modified equation would little different from those given by equan 6. It seems more likely that in fact and s vary from one species to another, t are not correlated with body weight. Equation 6 may be identified with the pirical equation,

$$Q_{O_2} = kW^{-p}. \qquad (7)$$

would have been equally possible to rt with equation (7), and to derive uation (3). By means of equation (7), d m values due to Brody, values of n ay be obtained from the graphs. They e shown in table 7, together with the rresponding values of p and m.

On the basis of some physiological as- mptions it is possible to get a rough idea n for several tissues, in order to see hether the n values in table 7 are reason- ble. It may be argued that under basal nditions *in vivo*, certain organs are per- rming their normal physiological func- ons in relation to the economy of the whole animal, and their metabolism should therefore be directly proportional to the B.M.R. For these tissues, n should therefore be approximately 0.73. These tissues are: the intestine, which absorbs what is metabolized; the liver, and all digestive glands; the kidney, which excretes the products of metabolism; the heart and respiratory muscles; and probably the skin, which gives off the heat produced. Direct proportionality with the B.M.R. would not be expected for the brain and reproductive organs. Lung weight and blood weight are an almost constant fraction of body weight. Since the bone marrow principally provides blood cells, its weight is very likely to be directly proportional to the blood weight, and hence to the body weight. The metabolism of lung and marrow should therefore be proportional to W, and n should equal one. Table 7 shows that for liver and kidney, n is close to 0.73, whereas for brain and lung, n is too far from 0.73 for the discrepancy to be due to errors in m and p. The rough agreement with the predictions from physiological considerations is satisfactory, since it is probable that none of the assumptions is strictly true.

Krebs concluded from his work on brain, liver, lung, kidney and spleen, that since the ratio of the Q_{O_2} to the B.M.R. per unit weight of the intact animal was greater the larger the species, there must be a major tissue whose basal Q_{O_2} was in approximately constant ratio with the B.M.R. per unit weight, and that this must be the striated musculature. The n value for muscle which would satisfy the equation $H = \Sigma dW^{-n}$ could be determined only if n were known for nearly all the other tissues. It cannot be estimated from Krebs' data, nor can his conclusion be confirmed, though it may be approximately true.

TABLE 7

Numerical constants in equations 1, 3 and 7, from graphs, and data of Brody ('45)

Organ	Medium	p (slope of graph)	m (Brody, '45 p. 591)	n (m − p)
Kidney	II	0.068	0.846	0.778
Liver	III	0.172	0.867	0.695
Brain	III	0.072	0.697	0.625
Lung	II	0.098	0.984	0.886
Spleen	II	0.133	not given	

Krebs also suggested that some change in the Q_{O_2} of a tissue might be expected, as body size increased, due to the increased proportion of supporting tissue and other accessory structures. It can be shown by taking a hypothetical extreme example, that this factor cannot account for much of the decrease in Q_{O_2} with increase of W. Suppose that in the mouse, an organ contains 1% of supporting tissue, of zero Q_{O_2}, which is distributed homogenously, and cannot be removed by dissection. Suppose that in the horse, this organ contains 20% of inactive tissue. The ratio for mouse to horse of the Q_{O_2} of the active tissue (i.e., all except the tissue of zero Q_{O_2}) will then be about 80% of the experimental Q_{O_2} ratio. The lowest such ratio found by Krebs was three for lung, and this would be reduced to 2.4, still leaving a large change of the Q_{O_2} of active cells to be explained.

The next question to be considered is whether Q_{O_2} *in vitro* is equal to Q_{O_2} *in vivo* for a tissue. The evidence given in table 5 for animals other than man, is inconclusive. The values in the table are not all equally accurate, and since the sums of regional metabolisms are not available for comparison with the B.M.R. the discrepancies cannot be checked. Where anesthetics were used, it may be doubted whether the animal was under basal conditions, and in some cases it seems unlikely that it survived the experiment in a normal physiological state.

However the inescapable conclusion from tables 2, 3 and 4, is that for most tissues, Q_{O_2} *in vitro* must be an over-estimate. If this is accepted, it would be desirable to know whether the discrepancy is due to overstimulation by the medium, or to some other experimental condition. Krebs' standard media were designed with the expressed intention of including all those substances which stimulate respiration. In particular, they contain three T.C.A. cycle intermediates at such concentration that a further increase causes no further increase of metabolic rate. This limiting total concentration is roughly 10 times the total concentration of all T.C.A. cycle intermediates, in serum. Most Q_{O_2} values determined in Krebs' media are higher than those previously reported for the same tissues in unsupplemented medi Krebs' results for brain cortex are co sistently higher than Elliott's in unsuppl mented media, although Pappius and E liott ('54) were not able to confirm th stimulating effect of T.C.A. cycle inte mediates. In general, there is therefor some ground for supposing that overstim ulation by substances in the medium partly responsible for the discrepancy, b the work of Pappius and Elliott sugges that experimental confirmation of th stimulation is desirable. Since it is b lieved that the T.C.A. cycle intermediate are not substrates but catalysts, ther would appear to be no reason against u ing them at serum concentration.

Part of the difference could be due t an imperfect imitation of the normal phy iological environment of the tissue. If th metabolism of the tissues were restricte in the intact animal by the rate of suppl of oxygen and substrates in the blood, an perhaps by their diffusion from the bloo into the cells, isolation of the tissues i media with a copious supply of their sub strates and oxygen should give highe metabolic rates.

Evidently the main effect of supple menting the medium is to increase k i the equation $Q_{O_2} = kW^{-p}$ (equation 7) i.e., to increase r in the relation Q_{O_2} *i vitro* = rQ_{O_2} *in vivo* (equation 5).

It can be shown that p may also b dependent on the medium. For example both Krebs and Elliott made comparison between the same series of brain corte slices in different media. Each autho used a pair of media which differed princi pally in that one contained calcium where as the other did not. The results of each worker, taken separately, indicate that p is independent of the addition of calcium However the value of p found by Elliott i significantly higher than that evaluated from Krebs' work. It is tempting to relate this discrepancy to the fact that Krebs media contained T.C.A. cycle intermedi ates, whereas Elliott's did not. However in Krebs' work on liver, in supplemented media, p is found to be dependent on cal cium concentration. On the basis of this evidence it is not possible to evaluate fully the effect of constituents in the media on the parameters of equation 7

the general relation between Q_{O_2} *in*
o and *in vivo* might be written:

$$Q_{O_2} \textit{ in vitro} = rQ_{O_2} \textit{ in vivo} \times W^t \qquad (8)$$

re r and t need not depend only on the
lium.

he r value for brain in table 4 is much
er than that for any other tissue studied
Krebs, although it might be expected
the stimulating effect of the medium
ld be about the same for all tissues.
s favors the suggestion made earlier,
the basal Q_{O_2} *in vitro* for brain should
hat for electrically activated tissue.

able 6 was devised as an illustration
ne difference in form between man and
fairly typical animals of very different
y weight. The major abnormality, but
the only one, is that man's brain is
ut 10 times the size of that of a "typical"
g animal. It has been demonstrated
the relation $Q_{O_2} = kW^{-p}$ (7) holds for
nan brain; consequently the relation
aW^{-n} equation cannot be true, and
seems less likely to be the equation of
damental importance than equation 7.
e equation 7 holds for man's brain,
organ most abnormal in size, it can
ccepted fairly readily for lung, spleen
liver which are also on the large side.
ther reassurance comes from a some-
t speculative argument on evolutionary
unds, that equation 7 is the funda-
ntal relation. Tissues develop from un-
erentiated cells which are probably of
ch the same size whatever the body
ght, W, of the mature animal. These
s contain genetic information as to the
l W, and their metabolism may also be
trolled genetically in accordance with
equation $Q_{O_2} = kW^{-p}$. As differentia-
into organs occurs, the Q_{O_2} increases
some groups of cells, and decreases in
ers, but homologous groups in different
cies are still related by equation 7. An
ivalent statement is that in mammals,
metabolism of the fetus is related
etically to the parental body size. An
mal may be born with an organ of
usual size, and if this alteration is
eritable, it may be selected if the
tabolism of the altered organ (probably
ler working rather than basal condi-
ns) is more appropriate to the environ-
nt than the metabolism of the usual
d organ. The existence of species for
which $Q_{O_2} = kW^{-p}$ holds, but $h = dW^{-n}$ does not, could in this way be explained.

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APPENDIX

Details of values and sources used in the compilation of table 2

Organ weights

Organ weights are based on a study of 7 sources. Detailed analyses of human cadavers have been given by Mitchell et al. ('45); Forbes et al. ('53); and also by Widdowson, McCance and Spray ('51) whose analyses of bodies "M" and "N" have been used here. Organ weights from post-mortem analyses of larger numbers of cadavers have been collected by several reviewers (Ross, '48; Sitsen, '31; Spector, '56, p. 162; Vierordt, 1888). The figures given in table 3 are not averages, but are selected typical values. Where one source gives a value inconsistent with the rest, that value has been ignored. Notes on the selection of values are given below.

1. All sources are in reasonable agreement for brain, liver, kidneys, pancreas.
2. Fewer values are available, and some are not consistent for adrenals, spleen, thyroid, thymus and testes.
3. For the lung, those detailed analyses, which were made up to a month after death, give values very high compared with the other sources. The lower values from analyses of fresh tissue have been preferred, since they are probably not complicated by pulmonary edema.
4. Figures for the skeleton are given by Mitchell et al. ('45); and Forbes et al. ('53). The estimate of the percentage of bone marrow in the skeleton is a guess, though the work on the marrow volume of guinea-pigs and rabbits has been considered. (Dietz, '44; Hudson, '58; Nye, '31).
5. The work of Mitchell et al. is the only source for adipose tissue, intestine and stomach and skin.

Tissue water

Few reliable figures are available. Those of Mitchell et al. ('45) and Forbes et al. ('53) have been taken for brain, liver, kidneys, intestine and stomach, skin, adipose tissue and whole skeleton. The figure of 22% for bone (excluding marrow) is based on the assumption that marrow is 70% water. The values for erythrocytes and plasma are well known (Spector, '56, p. 52). Muscle has been taken arbitrarily as 72% and other tissues, for which values are not available, have been assumed to be 70% water.

Q_{O_2} *values*

1. Brain, liver, kidneys, lungs and spleen: interpolated from graphs.

2. *Stomach and intestine.* Values of Q_{O_2} for rat tissues in bicarbonate-Ringer's and Glucose were determined on the basis of initial dry weights, by Dickens and Weil-Malherbe ('41). The estimate in table 3 is based on the following figures from their paper.

Mucous membrane from: jejunum, 15.6; duodenum, 8.8; ileum, 5.3; colon, 3.4. In another experiment, whole wall of jejunum, 7.8; mucosa, 11.3; muscularis, 6.3. In general quite large variations were found in the Q_{O_2} of the same tissue from different animals.

In relation to the above figures, 7 appears to be a reasonable value for the Q_{O_2} of rat intestinal tissues taken together. It seems likely that the Q_{O_2} of the human intestines should be lower, so 5 has been arbitrarily chosen. A value of 9.6 has been observed for human gastric mucosa (National Research Council, '58, p. 287).

3. *Bone marrow.* Bird and Evans ('49) found that the average oxygen uptake of suspensions of rabbit bone marrow cells in the pH range 6.9 to 7.3 was 3.6 mm^3/hour/mg of cell protein. This has been multiplied by 0.91 to convert to a basis of fat free dry weight.

4. *Skin.* Ohara ('51) determined the Q_{O_2} of pieces of skin taken from various parts of live human bodies. An average value for axillary skin was 0.93, whereas the values for samples from other regions were lower. A significant correlation was established between the Q_{O_2} and the volume percentage of sweat glands in the slices. The value of 0.6, taken as rep sentative of human skin on the basis this work, is somewhat lower than ot values to be found in the literature. (B ron et al., '48; Walter and Amersba '48).

5. *Adipose tissue.* Values for the a pose tissue of the rat are quoted below their original form (National Resea Council, '58, p. 287). In converting th into conventional Q_{O_2} values, all the sues have been assumed to be 23% wat

Brown fat: 0.419 mm^3 O_2/mg wet w hr.; Q_{O_2}, 0.54.

White fat: 0.049 mm^3 O_2/mg wet w hr.; Q_{O_2}, 0.064.

Retroperitoneal fat body: 7.9 μM O_2/ wet wt./hr.; Q_{O_2}, 0.23.

Value taken for man, 0.07.

6. *Adrenals, testis, thymus and p creas.* Values for rat taken (Spector, ' p. 260).

7. *Thyroid.* Values for cow, rabbit, d and rat (National Research Council, ' p. 289) were plotted against B.M.R. p unit weight. The value interpolated 70 kg is 9.

8. *Erythrocytes.* (National Resea Council, '58, p. 103.)

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eflex Relationships of the Abdominal Stretch eceptors of the Crayfish

FEEDBACK INHIBITION OF THE RECEPTORS[1,2]

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Since their description by Alexandrowicz 51) the dorsal abdominal stretch recepor organs of decapod crustaceans have rovided unusual opportunities for the inestigation of such basic neurophysiologial phenomena as sensory transduction, npulse generation, synaptic inhibition, ransmitter substance action, temperature ompensation, etc. (Eyzaguirre and Kufer, '55a,b; Kuffler and Eyzaguirre, '55; uffler and Edwards, '58; Burkhardt, '59). his was made possible by the ease of pplying physiologically normal stimulaion to the sensory neuron, as well as by ertain anatomical advantages, such as its elatively large size and the presence of an nhibitory innervation. It is the ease with hich the stretch receptor organs can be emoved from the animal and investigated *n vitro* that has, undoubtedly, delayed an nvestigation of their physiology *in situ.* ecause of the large amount of informaion gained from these receptors concerng events at the single neuron level, it was elt that an investigation of the physiology f the stretch receptors as a system would e of timely interest.

The two receptor muscles (RM_1 and M_2) of each pair were found to stretch rom the anterior end of one abdominal egment to the anterior end of the precedng (forward) segment at the median edge f the superficialis medialis muscle (see ig. 1). The muscles are innervated by a ranch, henceforth referred to as the dorsal nerve," of the dorso-lateralis nerve, hich has as its origin the second root of he ventral cord ganglion situated in the ext anterior segment (Wiersma et al., '53; ughes and Wiersma, '60). The dorsal erve is known to contain a number of motor and inhibitory fibers innervating the extensor muscles and the muscles of the stretch receptor organs, axons of the stretch sensitive neurons associated with the RM's, and a centrally originating fiber (Alexandrowicz's "accessory neuron") seen to branch and innervate the dendrites of both stretch receptor neurons. The dendrites of the receptor neurons embed themselves in the receptor muscles in such a way as to be physically distorted by stretch applied to the muscle (Alexandrowicz, '51; Florey and Florey, '55).

The anatomical arrangement of the receptor muscles and their innervation suggested that the stretching of the RM's during abdominal flexion could be a stimulus signal to the central nervous system via the sensory neurons in the dorsal nerve. Electrophysiological confirmation of this view was provided when Wiersma et al., ('53) recorded trains of afferent impulses during flexion of the isolated dorsal abdominal strip as well as upon stretching of single RM's *in vitro*. Electrical stimulation of the motor fibers demonstrated further that the relatively coarsely striated RM_1 responds more tonically to motor stimulation than does the finely striated RM_2 (Kuffler, '54). The motor responses of the two receptor muscles thus parallel their

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[1] This paper and the one that will follow (Eckert, '61) comprise part of a thesis presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Columbia University.

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characteristics as sensory systems—RM_1 adapts slowly while RM_2 is quick-adapting (Wiersma et al., '53). Of unusual interest was the demonstration by Kuffler and Eyzaguirre ('55) that the accessory neuron was indeed an inhibitor which suppressed sensory output from the stretch receptor dendrites by repolarizing their receptor membranes toward the resting level.

It seems apparent, then, that the sensitivity of the stretch receptors must normally be under a dual central control. Isometric contraction of the receptor muscles should increase the sensitivity of the receptor neurons in the intact animal because of the deformation of the receptor dendrites (Kuffler, '54). Any such deformation would set up a generator potential just as happens when the isolated muscles are experimentally stretched (Eyzaguirre and Kuffler, '55). On the other hand, the frequency of receptor discharge is known to be slowed, or even arrested, by discharge of the inhibitor neuron arising in the central nervous system. Additional modulation might, of course, result from neuromuscular inhibition or the action of blood-borne substances.

Under what circumstances, in the normal activity of the crayfish, is the sensory output of the stretch receptor modulated by centrally originating inhibition or potentiation? Is it the general central excitatory state that determines the degree of peripheral receptor neuron inhibition, or is the inhibitor reflexly activated as a consequence of sensory input? Isolated receptor preparations alone, cannot provide the answers to such questions. Only by retaining the nervous connections between the receptor organs and the central nervous system of the relatively intact animal can the normal reflex relationships of the stretch receptor system be investigated. Preliminary reports of the successful use of such *in vivo* preparations have already been made (Eckert, '60a,b).

Described below are a series of experiments designed to explore the relationship between the sensory output of the stretch receptor system and the modulation of that output via the central nervous system of the intact animal. Other reflex relationships of the stretch receptor system will be considered in the second paper of this series (Eckert, '61).

MATERIALS AND METHODS

Most experiments were conducted on *Procambarus blandingii blandingii* (Harlan), but a small number were performed on an undescribed species of *Cambarus* (Hobbs, personal communication). No specific differences were noted. Stocks were maintained at about 15°C, and were replenished from a commercial source at approximately three week intervals. The shallow water containing the animals was changed at frequent intervals to prevent clouding or accumulation of waste. Animals thus maintained showed a relatively low mortality rate, and retained a high degree of normal behavioral response.

In setting up the experimental preparation, the chelipeds and walking legs were first removed from the crayfish. By means of a scalpel and scissors, the dorsal musculature of several segments was exposed by careful removal of all but the anterior portions of the terga (see fig. 1). This simple operation lays bare the receptors and their innervation without injury to either. Equally important, the anatomical relations of the receptors to the rest of the abdomen are maintained, and there is no interference with functionally normal receptor stimulation by tail flexion. The animal was then suspended dorsal side up, held by a carapace clamp, in a physiological saline based on Van Harreveld's ('36) solution. The bicarbonate was omitted from this saline, and the remaining salts were dissolved in 0.01 M tris-maleate buffer, giving a final pH of 7.2. This modification allowed oxygen saturation of the solution without a concomitant change in pH. Because of the fall in blood pressure resulting from the operation, and the sensitivity of crayfish synapses to anoxia (Wiersma and Garcia Ramos, '53), the saline was oxygenated prior to the experiment. The solution around the operating chamber was maintained, for the same reason, at 6–8°C by means of an ice jacket. In spite of these precautions the preparation usually deteriorated in less than one hour. An additional advantage of low temperature is its anesthetizing effect, which reduces the motor activity of the animal.

In the various experiments described elow, action potentials were led off by neans of platinum hook electrodes of .005 inch diameter. Amplification was y means of Grass P5 capacity-coupled reamplifiers. Indifferent platinum electrodes were placed in the saline bath, vhich also was grounded. Nerve impulse otentials were photographed from a dual hannel cathode ray oscilloscope, and nonitored by means of a second CRO and oudspeaker system. A Grass S4C stimulaor provided square wave pulses in experinents where electrical stimulation was sed to confirm results of mechanical timulation. During stimulation and reording the electrodes and associated erve bundles were exposed to the air by iphoning off some of the saline, while etween recordings the structures were gain immersed to prevent undue dehydraion.

Although some experiments were perormed with the RM's *in situ,* it was usually desirable to isolate one experimental pair of receptors from mechanical disturbances of the abdomen while still maintaining intact neural connections to the central nervous system. This was accomplished by clamping the ends of the receptor muscles in a pair of jig-mounted watchmaker's forceps before cutting away the muscle insertions. By means of a micromanipulator the RM's were then carefully moved laterally away from the abdomen while the branches of the dorsal nerve innervating extensor muscles were severed, allowing the RM's to be well isolated from the abdominal musculature, yet retaining their central connections (see fig. 1). The tension on the RM's could then be varied by adjusting the relative position of the two forceps. Either of the receptor muscles could also be stretched by freehand manipulation of a fine needle while recording from the appropriate nerve bundle. The other RM's could be individually stimulated by stretching with

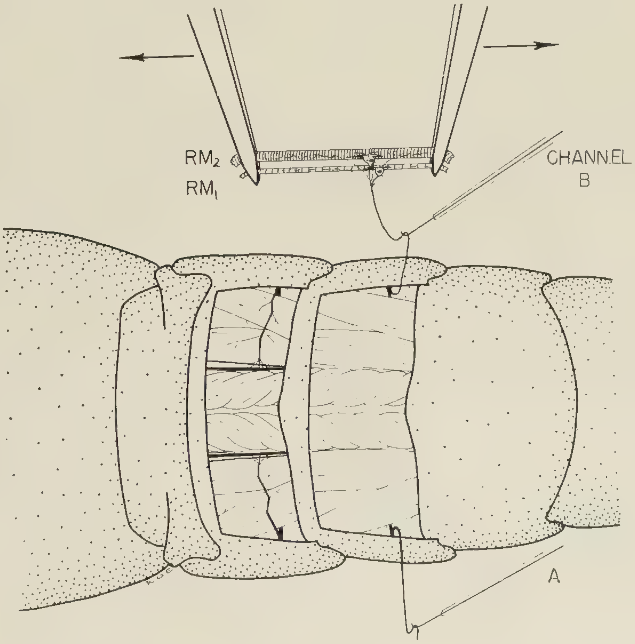

Fig. 1 Manner of RM isolation retaining intact the connections with the central nervous system. The two receptor muscles (drawn oversized) are held by mechanically mounted forceps, so that they can be stretched or relaxed and so maintained as desired. The receptor output is monitored by electrode "B," while centrally originating impulses of neighboring dorsal nerves are monitored by electrode "A." The natural positions of the receptor organs and their innervation can be seen in the exposed second segment.

a probe, or they could be collectively stimulated by manually flexing the tail.

RESULTS

1. *Efferent fiber response to RM stimulation*

When a dorsal nerve of an intact crayfish is exposed by means of a "window" cut into the dorsolateral portion of the tergum of an anterior abdominal segment, it is possible to record stretch receptor output by placing a recording lead under the intact nerve bundle. Subsequent manual flexion of the abdomen is accompanied, as expected, by an increase in the discharge frequency of RM_1. Upon strong flexion the larger RM_2 spike appears. Because of the large diameter of the sensory neurons, it is possible to record their activity even though the nerve cannot be pulled out of the body fluids to any great extent under these conditions. Smaller fiber activity, however, is largely lost in the noise of the recording system in this experiment. In order to facilitate observation of efferent activity, the dorsal nerve was therefore cut distal to the electrode and lifted out of the body fluid, thus severing its connections with the stretch receptors. Rather unexpectedly, it was observed that under these conditions (no direct connections with receptors) there was a recorded response (fig. 2A) to passive (manual) tail flexion, evidently in fibers having a central origin.

This efferent response can be seen to represent three fibers: (1) a tonically active neuron producing relatively low amplitude spike potentials (ca. 0.02 mv by this recording method), just visible above the noise level and increasing in frequency as the tail is flexed; (2) a larger fiber (ca. 0.25 mv) responding more phasically to tail flexion; and (3) an even larger fiber (ca. 1 mv) discharging at a lower, irregular frequency to tail flexion and extension. It was thought that the most likely sources of these central responses to tail flexion were the remaining stretch receptors or the proprioceptor hairs located at the joints of the cuticular covering of the abdominal segments.

Subsequent denervation of all abdominal RM's indicated that fibers 1 and 2 depend for their response on the integrity of the

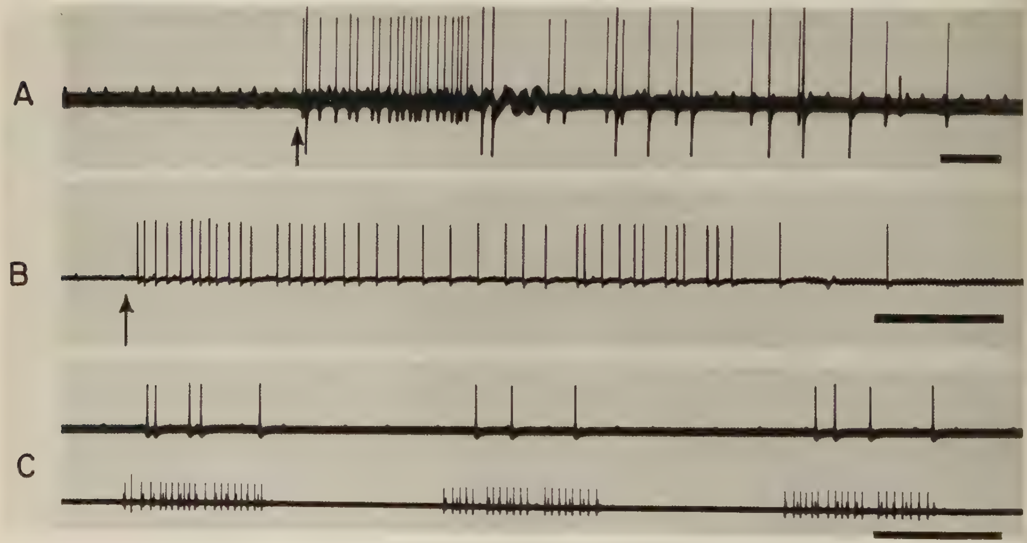

Fig. 2 A, efferent impulses recorded in dorsal nerve in response to passive tail flex (arrow). Note the three characteristic spike potentials described in the text. Because of the amplification required to reveal the smallest spike, the tops of the largest of the three potential sizes have been cut off. B, response to passive tail flexion by the smaller phasically discharging efferent fiber. C, the same fiber (upper trace) responding to the afferent discharge of the intermittently stretched contralateral stretch receptor pair (lower trace). Time marks represent 0.5 second in each record. Spike voltages are discussed in the text.

retch receptor system, since the two wer amplitude potentials were never seen respond to tail flexion after this opera- on. The response of the largest fiber (3, bove) is unchanged following this opera- on and therefore must depend upon some ther sensory mechanism, such as the pro- rioceptors of the abdominal joints. The emainder of this paper will deal with the ber (2, above) that responds phasically stimulation of the stretch receptor sys- em (fig. 2B).

2. *Convergence of RM_1 and RM_2 outputs upon the same efferent neuron*

It was important to determine whether his fiber is dependent upon the summed ctivity of a number of receptor pairs, or f stretching one pair of RM's is sufficient o evoke the response. By means of a fine eedle a pair of intact RM's was stretched while their response was recorded along vith the efferent activity of a dorsal nerve. The resulting response can been seen in igure 2C. The finding that a single RM pair could elicit efferent spikes greatly implified subsequent experiments, for it vas then possible to activate the efferent iber under more precisely controlled con- litions than are possible during gross stimulation by tail flexion. For example, n order to determine whether or not both RM_1 and RM_2 evoke the same response, a pair of receptors was semi-isolated as shown in figure 1. While recording efferent activity of a nearby dorsal nerve, first RM_1 and then RM_2 of the isolated pair were individually stretched in order to compare responses. It is evident from figure 3 that accompanying sensory discharges from either RM_1 or RM_2 there was a train of spikes in the same efferent axon. This result indicates that the afferent sensory impulses from both receptor organs must converge, directly or indirectly, upon the same efferent neuron. It was generally noted, however, that the sensory impulses from RM_1 were more effective than those from RM_2 in activating the common efferent pathway.

3. *Identity of the efferent neuron reflexly activated by stretch receptor output*

From which efferent fiber are impulses being recorded during stimulation of the receptor organs? Efferent fibers of the dorsal nerve are known to include: (1) extensor motor fibers; (2) extensor inhibitors; (3) and (4) similar fibers to the stretch receptor muscles, at least some of which the RM's share with the extensor muscles (Florey and Florey, '55); and (5) the inhibitor fiber impinging on the dendrites of both receptor neurons.

Absence of an efferent response to RM stretch while recording from extensor

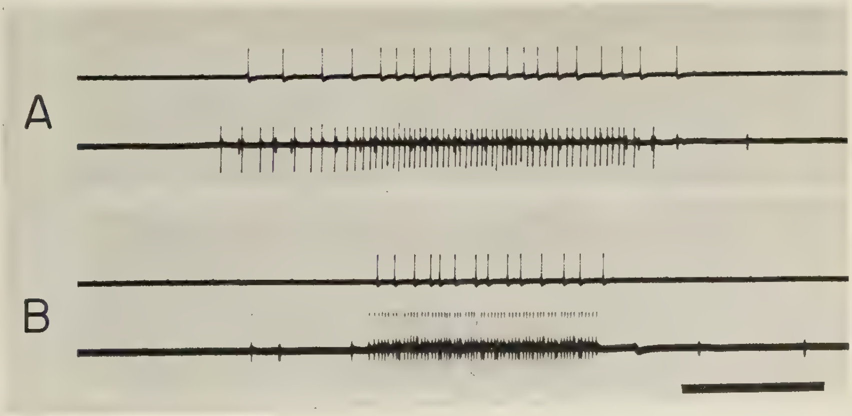

Fig. 3 Central convergence of RM_1 and RM_2 on the same efferent neuron. A, efferent fiber responding (upper trace) to sensory discharge of a stimulated RM_1 (lower trace) of an adjacent segment. B, same fiber firing during RM_2 discharge in the same preparation. Note that a higher RM_2 frequency is characteristically required to evoke the same reflex firing frequency. Time mark represents 0.5 second.

muscle branches of the dorsal nerve provided evidence that the fiber in question is not an extensor excitor or inhibitor. Furthermore, the subsequent observation that an efferent response could be detected at that portion of the nerve entering the RM's lent support to the hypothesis that the impulses returned to the receptor organs in a feed-back-like manner, or, stated differently, that stretch receptor output was indirectly modulating itself by activating the efferent innervation of the receptor organs. Stretch receptor output could be either reinforced by contraction of the receptor muscles, or lowered by inhibitor nerve activity.

Which of these two possible mechanisms of feedback modulation was actually occurring? To distinguish between these two possibilities a preparation was set up as pictured in figure 4. Two electrodes, connected to the CRO via two separate recording channels, were so manipulated that one gently stretched an intact pair of RM's (A) by their nerve so as to record the steady train of RM_1 spikes in response to the stretch; while the other electrode monitored the output of a semi-isolated RM_1 (B) as it was in turn stretched and relaxed several times by means of a fine needle. It was reasoned that if the resulting sensory output from the semi-isolated RM_1 centrally activated the intact RM motor fibers an increase in the discharge rate of the intact receptor (A) should result, since contraction of the RM's of the adjacent segment would stimulate their sensory neurons. To the contrary, however, it was found that discharge of receptor B was accompanied by an interruption of the discharge train of receptor A (fig. 5A). This strongly suggested that it is the inhibitor fiber ("accessory fiber" of Alexandrowicz) which is centrally activated by stretch receptor discharge.

The obvious relationship to be seen between the small efferent spikes and the inhibition of RM discharge (figs. 5A, C, and D) lent further support to the suggestion that it is the inhibitor to the receptor dendrites which is reflexly activated by RM stimulation. Moreover, the timing of the inhibitory impulse with respect to that

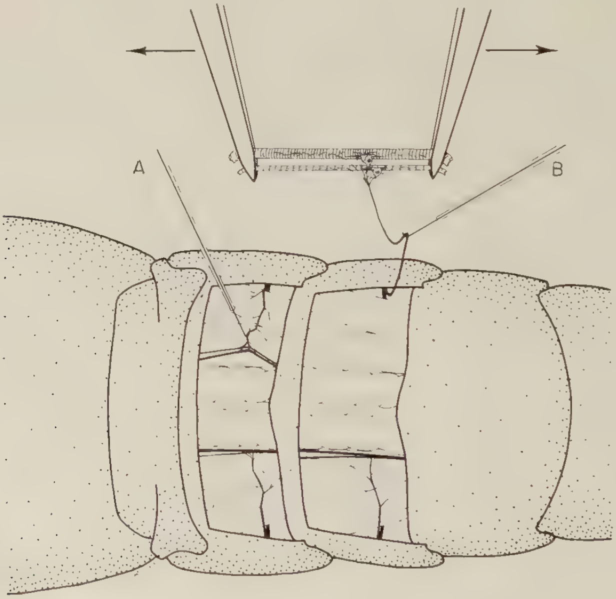

Fig. 4 Experimental arrangement used to determine the identity of the reflexly activated efferent fiber. Electrode A is hooked under dorsal nerve so as to slightly stretch the RM's of segment 2 while recording the RM_1 discharge train in response to the steady stretch. Electrode B monitors for a second channel the receptor output resulting from intermittent stretching of the isolated RM pair of segment 3.

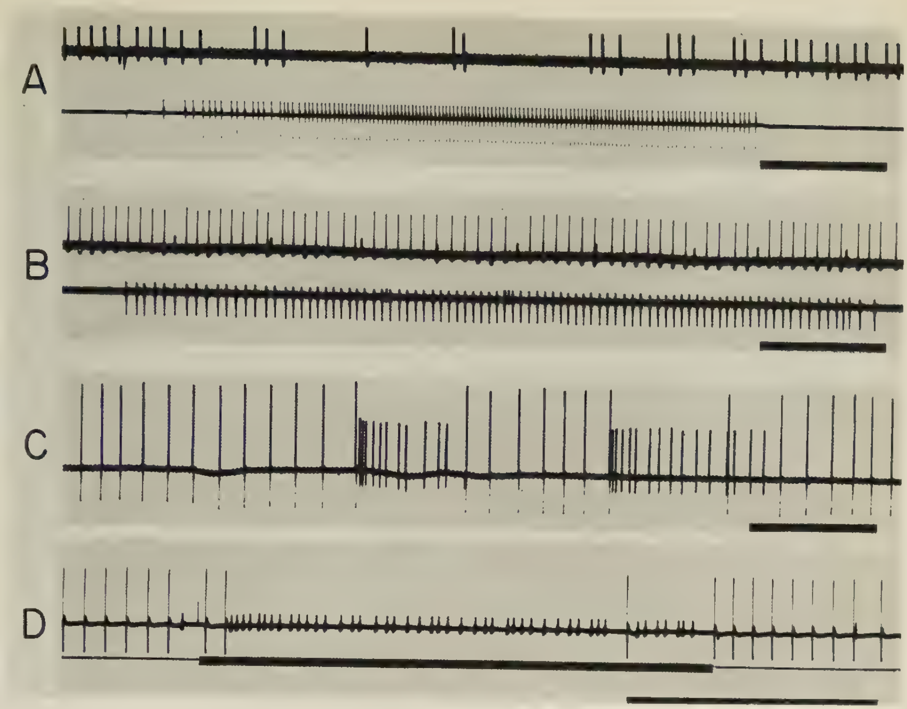

Fig. 5 A, regular firing rate of a steadily stretched RM_1 (upper trace) interrupted by stimulation of a neighboring RM_1 (lower trace). See figure 4 for experimental conditions. B, conditions similar to those of A. Small spikes in upper trace are clearly associated with the inhibition of RM_1 spikes. C, similar to the two previous records except that recording conditions were reversed so as to record the tonic output of an isolated RM_1 during intermittent stretching of an intact RM pair in an adjacent segment. As in upper records, the stretching is followed by the appearance of inhibitory impulses and interruption of RM_1 output. For technical reasons it was not practical to include a second trace for the output of the stimulated *in situ* receptor pair. B, train of RM_2 impulses (upper trace) inhibited by electrical stimulation (lower trace) of dorsal nerve of the contralateral side. Mark on lower trace indicates the duration of the electrical stimulation (100 pulses per second), although individual pulses do not show. Some spikes retouched for contrast. Time marks represent 0.5 second.

f the sensory impulse appears to be of im- ortance in determining its effectiveness fig. 5B). If the recorded inhibitory spike rrives just after one of the sensory spike otentials, the next sensory spike in the ain will be delayed by a small amount; owever, this delay increases as the posi- on of the inhibitory spike approaches 1 time the position of the next sensory pike as it would normally occur in the bsence of inhibition. With optimal tim- ig, one inhibitory impulse can almost ouble the time interval between two pikes of a train. A train of inhibitory npulses of sufficient frequency will, in act, prevent any receptor discharge for ne duration of inhibitor discharge (fig. C, D).

Although the evidence was strong in favor of the theory that it is the accessory fiber that is responsible for the reflex inhibition of stretch receptor output, there remained the possibility that it is, instead, the discharge of a *motor* inhibitor fiber that is involved in reflex inhibition. If the muscle were under tonic excitation, such neuro-muscular inhibition would result in relaxation of the muscle and subsequent reduction of tension, causing the discharge frequency to drop. That the inhibition is actually taking place at the sensory cell itself was confirmed by recording the inhibitory postsynaptic potentials accompanying reflex inhibitory spikes. A slow potential record of postsynaptic events was obtained by placing one recording lead directly on the receptor cell while employing wide band A.C. preamplification. It was seen that the inhibitory impulses (elicited by stretching the contralateral receptor) drive the membrane potential in a direction opposite to that of the generator potential of the sensory cell; that is, the efferent impulses hyperpolarize the post-synaptic membrane under the experimental conditions. This was found by Edwards and Hagiwara ('59) to be the case when the sensory inhibitor was firing, and may be considered (Edwards, personal communication) to be a rigorous criterion for identification of synaptic sensory inhibition in this preparation.

It is observed (fig. 5D) that RM_2 as well as RM_1 is inhibited by the efferent fiber which is reflexly activated by stimulation of either kind of receptor. Histologically, the sensory inhibitor fiber is seen to branch as it approaches the receptors, innervating the dendrites of both the slow and fast receptor cells (Florey and Florey, '55). The anatomical data thus correlate with, and provide an explanation for, the observation that both receptors are reflexly inhibited.

4. *Relative degree of interaction of receptors located in different segments*

In the majority of these experiments feedback observations were restricted to one or two segments at a time as a matter of experimental convenience. For example, a second segment receptor was stretched while efferent impulses were recorded in the contralateral nerve or in the nerves of segment 1 or segment 3. Does similar interaction between receptors take place when they occur in widely separated segments? To answer that question, receptors in segments 1–4 were exposed in the usual manner, and their nerves cut near the receptors. A recording lead was hooked under one of the nerves while, one at a time, the remaining dorsal nerves were stimulated by means of two hook electrodes delivering 1.5 millisecond pulses of 4 volts at a rate of 100 per second. In this manner the sensory fibers of each exposed RM pair could be reproducibly stimulated while the degrees of inhibitory feedback elicited in the monitored nerve were photographically recorded and compared. The segmental location of the monitored nerve was varied in different experiments and was found to be unimportant to the general picture that emerged. On the basis of complete experiments on 4 such preparations, and additional observations on other preparations, the following general conclusions are possible: (1) the intensity of feedback originating from any one receptor pair clearly diminishes in degree with the number of segments that separate the interacting receptors; (2) stimulation of one pair of receptor neurons does not evoke efferent activity more than three segments away; (3) although one side is often more effective than the other in eliciting inhibitor activity, neither homolateral nor heterolateral stimulation is consistently more effective than the other in comparisons between different crayfish; however, (4) when stimulation of one side is more effective, this difference is consistent for all the investigated segments of that crayfish. These observations have implications concerning the nature of the central connections that must exist between the stretch receptor axons and the receptor inhibitor fibers, and will therefore be considered at greater length in the discussion section.

Since the discharges of receptors as far as three segments away evoke a discharge in the same inhibitory fiber, considerable anatomical convergence must be present. Does this convergence manifest itself, as might be expected, in a spatial summation of receptor discharge? This problem was explored in 4 preparations by recording from one dorsal nerve while stimulating electrodes were placed on two other nearby dorsal nerves. Electrical stimulation at frequencies ranging from 10 to 100 pulses per second were applied first to one nerve, then the other, and finally to both nerves simultaneously (this order being randomly varied in successive observations). Figure 6 shows that simultaneous stimulation of nerves A and B results in an inhibitor response much greater than the summed responses to separate stimulation. This suggests that facilitation occurs at one or more synapses of the feedback pathway. Since the RM's are simultaneously stretched during the swimming reflex, this

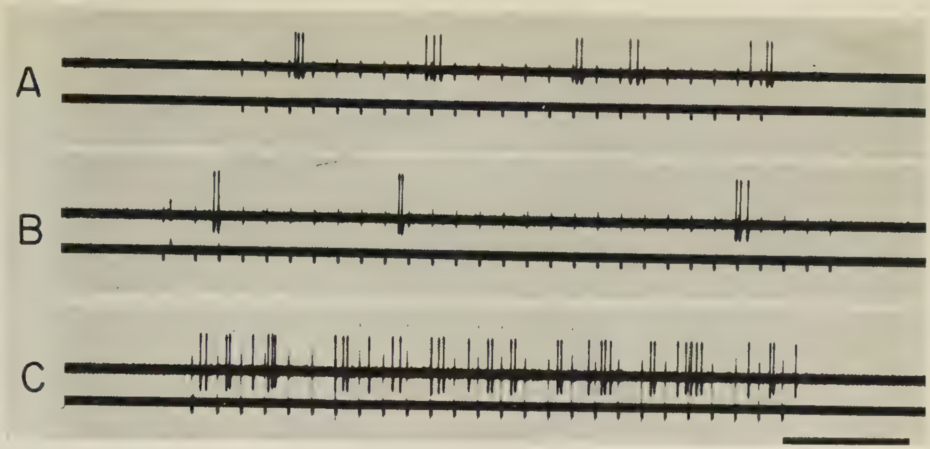

Fig. 6 Spatial summation of receptor axon discharges. A, inhibitor fiber response (upper trace) to electrical stimulation (10/second) of dorsal nerve in an adjacent segment. B, same fiber responding to similar stimulation of the contralateral dorsal nerve. C, simultaneous stimulation of both nerves results in a greatly facilitated response of the inhibitor neuron in the third dorsal nerve. Time mark represents 0.5 second.

kind of summation of receptor activity and the resulting increase in inhibitor discharge would appear to be of considerable functional significance.

5. *Feedback inhibition of a single stimulated receptor*

The finding that inhibition decreases in relationship to the spatial separation of the interacting receptors is reminiscent of sensory contrast mechanisms of such systems as the lateral eye of *Limulus*, and the mammalian eye, somatic sensory cortex, and stretch receptor reflex (Hartline et al., '56; Kuffler, '52; Mountcastle, '57; Brooks and Wilson, '58). Does the reflex inhibition of the stretch receptor system similarly act to enhance the sensory "contrast" of flexion among abdominal segments? If contrast is indeed the function of this phenomenon, it would be necessary that each receptor inhibits only its neighboring receptors and not itself, or it must at least inhibit the other receptors to a greater degree than it inhibits itself. If on the other hand, the receptors inhibit themselves as much or more than they do adjacent receptors, a contrast role would be most unlikely.

In previous experiments it had been noticed that the stimulation of a stretch receptor does indeed result in a discharge in the inhibitor innervating that receptor. In order to determine the relative degree of autoinhibition, a preparation similar to that in figure 1 was used, permitting the recording from a single nerve of both afferent and efferent impulses associated with RM_1 stretch at the same time that a second channel monitors the activity of the inhibitor fiber to the receptor in an adjacent segment. After photographically recording the responses to RM_1 stretch, the inhibitory spikes in both channels were counted and compared. There were no significantly consistent differences in numbers of inhibitory spikes going to the initiating receptor or to one of its adjacent receptors in the ten experiments performed. In some cases the frequency of response in both inhibitors was about equal, while in some preparations the initiating receptor received more, and in others received fewer inhibitory impulses than its neighboring receptor. Evidently, the reflex inhibition of the stretch receptor system does not function to enhance proprioceptive contrast.

6. *Central pathways of the feedback arc*

Related to the question of the spatial interaction of the receptors is the problem of the central pathways and connections that form the basis for the physiological observations. How many ganglia, for ex-

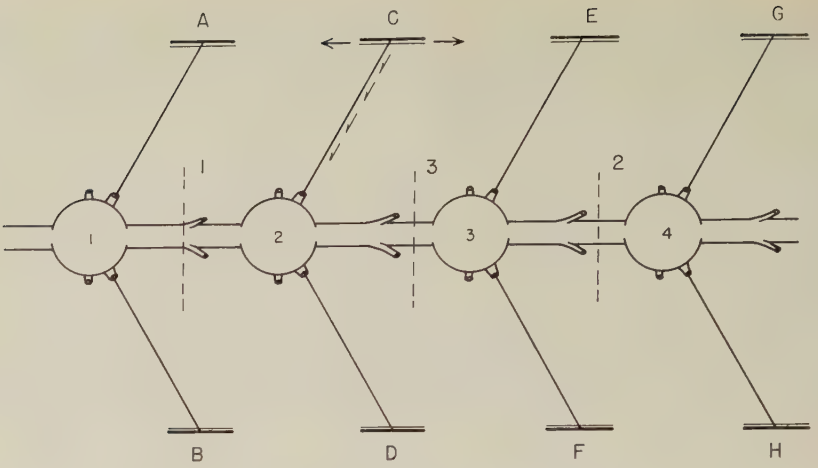

Fig. 7 Schematic diagram of several ganglia (1–4) with associated stretch receptor pairs (A-H). Text describes the results of cutting at levels 1, 2, and 3 on inhibitor activity evoked by stimulation of receptor pair C.

ample, are involved in the interaction of any two receptors of the same segment? A preliminary approach to such problems is illustrated in figure 7. A pair of RM's (C) could be stretched so as to evoke inhibitory fiber activity in surrounding dorsal nerves A, B, D, E, and F. The cord was then severed at level 1. This, obviously, eliminates communication with receptors A and B, but also diminishes somewhat (or sometimes eliminates completely) the frequency of inhibitor impulses in nerve D. If the cord is subsequently cut at level 2, there are no further pronounced effects upon interactions of C, D, E, and F. However, the cut at level 3 completely eliminates the remaining inhibitory interaction between receptors C and D, even though their ganglion remains intact with them. Although this is certainly not conclusive evidence, it does seem to indicate that connections between receptors and inhibitors are made in the ganglia anterior and posterior to the ganglion that the receptor neuron enters, and that no direct connections exist within a ganglion between the receptor and inhibitor neurons of that ganglion. This is not in disagreement with the results of physiological and histological investigations, by other workers, of the central pathway of the RM sensory neurons. After entering the ganglion, the sensory fiber was found to bifurcate so that one branch passes uninterrupted to the brain, while the other branch synapse with a common interneuron in the las abdominal ganglion. Moreover, histological examination failed to disclose any collaterals in the initial ganglion (Hughe and Wiersma, '60).

In order to determine whether such collaterals can be seen in the ganglion anterior and posterior to the initially entere ganglion, transverse sections of the crayfish cord were made according to Johnson's ('24) method. Unfortunately, it doe not seem possible, upon examination o the sections, to determine with any degree of certainty the identity of the sensor fiber as it enters the ganglion an branches. This has made it impractical t determine, histologically, the presence o absence of collateral branches. It seems certain, however, from the physiologica experiments that local synaptic connections of some kind must exist in the ganglia in order to account for the reflex activation of the inhibitor.

DISCUSSION

By means of a preparation that retains intact the central nervous connections o the abdominal stretch receptors, it has been possible to observe a reflex inhibitio of the sensory response to tail flexion. The results indicate that the stretch recepto system is not merely an aggregation of independently reacting receptors, but intead exhibits an extensive degree of receptor interaction. That is to say, in addition to feedback inhibition of itself, each active receptor contributes to the inhibition of receptors in neighboring segments. This is apparently accomplished by connections, in several ganglia, between a given sensory neuron and the inhibitor neurons known to impinge upon the dendrites of that stretch sensitive neuron and those stretch receptors of nearby segments. Since histological methods have thus far been unable to elucidate the nature of such central connections, it is necessary to rely on physiological observations for an understanding of the nervous pathways involved in this phenomenon.

An attempt to visualize a simple scheme of central connections in agreement with the functional findings is seen in figure 8. Receptor organs (e.g., RM_1's) of one side of 4 consecutive segments (1–4) are pictured at the top of the schematic diagram. Each is associated with a sensory axon that, upon entering its respective ganglion, bifurcates, sending one uninterrupted branch anteriorly (left) and one posteriorly (right) to both extremities of the central nervous system (Hughes and Wiersma, '60). Near the lower portion of the figure the inhibitor neurons are seen as broken lines, while the hypothetical interneuron system is seen to lie between receptor and inhibitor neurons.

The following physiological observations make it necessary to postulate such an interneuron system: (1) The degree of inhibitor activity elicited by stimulation of a receptor decreases as the number of segments lying between receptor and inhibitor is increased. A longitudinal series of synapses, each diminishing the presynaptic frequency, best explains this segmental decay. (2) A given inhibitor fiber will often respond more intensely to stimulation of receptors of one side than to those of the other side. Because this is unpredictable and not consistently related to the side containing the inhibitor being recorded from, it is likely that this lateral response imbalance results from chance differences in synaptic connections between the inhibitor and elements common to the receptors of each side. The element common to a side is most likely an interneuron system. (3) Although there is no evidence for a monoganglionic receptor-inhibitor pathway, and in fact, some evidence against it, the receptors of a segment are seen to excite the inhibitor fibers of the same segment. This suggests that the connections between receptor and inhibitor neurons of one ganglion involve pathways through one or both adjacent ganglia. This seems best explained by assuming the presence of an interneuron between the inhibitor neuron and a receptor axon collateral arising in the adjacent ganglion.

Figure 8 is undoubtedly only one of several nervous schemes that might fit the physiological observations; however, it is one of the simpler possible schemes. It can best be understood by following the consequences of stimulating a single receptor, for instance, receptor 2. A train of impulses passes anteriorly and posteriorly on the two branches of the sensory axon, entering the collaterals in segments 1 and 3. By means of synaptic junctions, interneurons B and C are then caused to fire—probably at frequencies lower than those of the sensory fiber. Interneuron B impinges directly on the inhibitors of segment 2 and the inhibitors of the segment anterior to segment 1 (not shown in diagram). Interneuron C synapses directly with inhibitors in segments 3 and 1. Also, interneurons B and C mutually excite each other, possibly reinforcing the sensory input. The physiological observations indicate that segments 1, 2, and 3 receive inhibitor stimulation of similar intensity when receptor 2 is stimulated, and that the inhibitors of the other segments are activated inversely proportionally to distance from segment 2. This is explained by assuming that the postsynaptic frequency is lower than the frequency of presynaptic impulses at each synapse. The inhibitors of segment 4 would thus be less strongly stimulated than those of segment 3 by output of receptor 2, since the information would have to pass through at least one more synapse to arrive at the segment 4 inhibitors. Still further reduction of stimulation would occur for the same reason before the information reached segment 5.

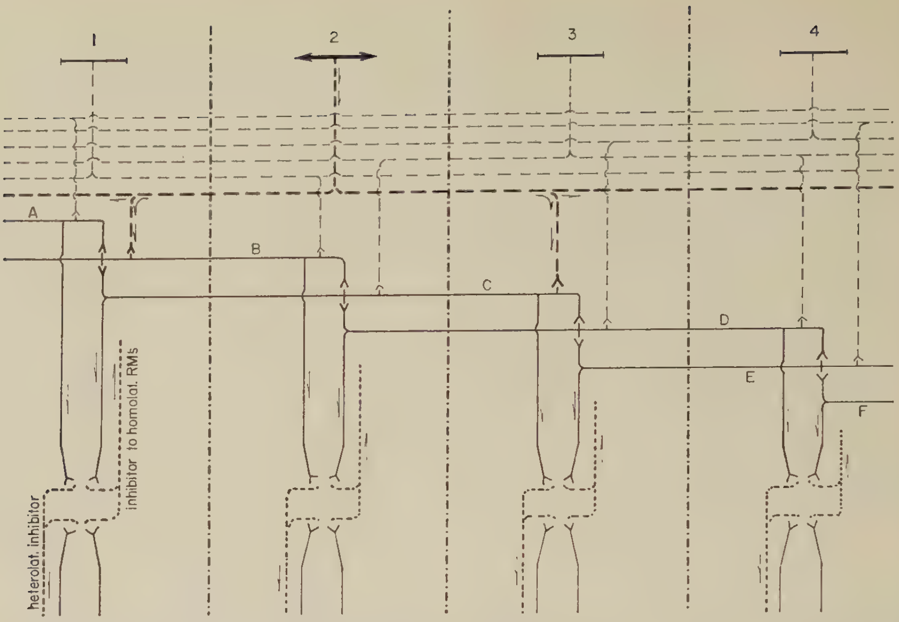

Fig. 8 A hypothetical scheme of central connections involved in the inhibitory feedback arc of the stretch receptor system. The divisions bounded by the vertical —·—·— lines represent neural segments associated with ventral ganglia. Interneurons are represented by solid lines; primary sensory neurons by dashed lines, — — —; and inhibitor neurons by broken lines, - - - -. Stretch receptor organs are represented at the top of the figure by horizontal solid lines associated with their respective sensory axons. See text for detailed explanation of the figure.

There are a number of possible pathways between receptor 2 and, for instance, inhibitors of segment 4—e.g., interneurons C-D-inhibitor; interneurons C-D-E-F-inhibitor; interneurons C-D-E-D-inhibitor; etc. However, there must obviously be a point in such a series of integrating synapses where the presynaptic frequency is insufficient to evoke a postsynaptic impulse. By arbitrarily designating the maximum number of effective synapses at 4, and determining the number of pathways by which each inhibitor can be excited during receptor 2 stimulation it is possible to determine the relative effects of receptor output on different inhibitors. Thus, the inhibitors of ganglia 1, 2, and 3 each receive receptor 2 output via one pathway involving two synapses, two pathways involving three synapses each, and one or two pathways involving four synapses. Inhibitors of ganglion 4 receive one pathway utilizing three synapses and another of four synapses, while the unpictured inhibitor neurons of ganglion 5 are weakly excited by one pathway involving four synapses.

Portions of the heterolateral interneuron system can be seen at the bottom of the diagram. In this scheme each inhibitor makes synaptic contact with interneurons of either side. Chance anatomical differences in the inhibitor's synaptic junctions will determine whether or not receptors of one side excite it more than do the receptors of the other side.

The foregoing hypothetical neuron map implies, among other things, that portions of an interneuron normally conduct impulses in both directions. Hughes and Wiersma ('60) have, in fact, observed this to happen in interneurons of the crayfish cord, in which case the colliding impulses cancel each other. This would serve to modify somewhat the number of impulses "getting through" to the inhibitor fibers. The suggestion that a single interneuron receives inputs from receptors in several segments is also consistent with the findings of other workers (Wiersma et al., '55).

Although the crustacean stretch receptor system exhibits peripheral sensory inhibition, such a mechanism has not yet been seen to exist in the mammal. Instead, inhibition of heteronymous reflexes (by "Renshaw cells") helps, in the mammal, to confine stretch reflexes to the path of afferent origin (Brooks and Wilson, '58). Similar to the crustacean, on the other hand, the mammalian stretch receptor system is known to possess an excitatory innervation in the gamma efferent system that brings about contraction of the intrafusal muscle fibers. Although modulation of the mammalian stretch receptor is under tonic control of central nervous centers, as well as under reflex control (Livingston, '59), only the latter mechanism has thus far been observed in the control of sensory inhibition of the crustacean stretch receptor system. It is possible that further studies may reveal that the discharge rate of the crustacean "accessory fiber" is also under the tonic control of higher nervous centers.

Arthropod nervous systems have contributed much in the past to our as yet fragmentary understanding of nervous mechanisms. The means employed by these systems are often novel and surprising when first uncovered. Certainly, a receptor system which to some degree automatically modulates its own output by inhibitory feedback gives rise to a number of problems. Possibly the most interesting question is concerned with the functional significance of feedback inhibition of sensory output in this system. The superficial similarity of this inhibitory interaction with that found in the contrast mechanisms of certain other sensory systems has been noted. It is not surprising, however, to find that this cannot be the functional significance of inhibition in this system, for it would be difficult to understand the necessity for such a mechanism in a tail that is, during swimming motions, made to flex as a unit by means of giant axon all-or-nothing neuromuscular triggering. Part II of this study (Eckert, '61) is concerned, in part, with a possible explanation for the functional role of feedback inhibition from stretch receptors during normal behavior of the animal.

SUMMARY

1. A preparation is described which makes it possible to isolate single crayfish stretch receptor organs for selective stimulation without destruction of their central connections.

2. Stimulation of single slow or fast stretch receptors, as well as flexion of the tail, gives rise to an efferent response in two fibers of the nerve innervating the stretch receptors and superficial extensor muscles.

3. One of these reflexly discharging fibers was shown to have an inhibitory effect on the output of both fast and slow stretch receptors. Inhibitory postsynaptic potentials recorded during this inhibition indicate that the reflexly activated fiber is identical with the inhibitor neuron known to impinge on the receptor cell dendrites.

4. Reflex discharge of the inhibitor can be elicited by stimulation of single receptors of the same abdominal segment or other receptors as far as three segments away. The effect diminishes as receptor and inhibitor lie in more widely separated segments. However, although the receptors of one side are often more effective in eliciting discharge of any one inhibitor fiber, there seems to be no consistent difference between homolateral and heterolateral reflex inhibition.

5. In view of the fact that any one receptor appears to arouse reflex inhibition of itself, similar in degree to that which it elicits in neighboring receptors, it is felt that the phenomenon does not enhance "contrast" between afferent sensory information from different abdominal segments.

6. Although the central pathways for this reflex are not as yet certain, sectioning experiments suggest that even the simplest stretch receptor-inhibitor pathways involve a minimum of two abdominal ganglia.

7. A hypothetical scheme of central connections, in agreement with the physiological observations, is suggested for this reflex.

8. It is concluded that the abdominal stretch receptor system of the crayfish possesses an inhibitory feedback mechanism which reflexly inhibits, to a degree, the intensity of stretch receptor discharge. This mechanism apparently involves central connections, via interneurons, between stretch receptor cell axons and the well-known "accessory" fiber of Alexandrowicz.

ACKNOWLEDGMENTS

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Reflex Relationships of the Abdominal Stretch Receptors of the Crayfish

II. STRETCH RECEPTOR INVOLVEMENT DURING THE SWIMMING REFLEX[1,2]

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A consideration of the morphology and physiology of the crustacean stretch receptor reveals a considerable degree of functional complexity (Alexandrowicz, '51; Wiersma et al., '53; Florey and Florey, '55; Kuffler, '58). In addition to possessing a sensory neuron capable of signaling stretch applied to the receptor muscle, each muscle receptor organ receives several efferent fibers. These, presumably, make possible neural modulation of receptor sensitivity. One of these efferent fibers, the inhibitory "accessory fiber" ending on the sensory dendrites, was shown to be reflexly excited by stretch receptor discharge (Eckert, '61). The remaining efferent fibers are seen to innervate the receptor muscle bundles, and presumably consist of both motor excitor neurons and motor inhibitor neurons.

The functional complexity of the stretch receptor system suggests that it is intimately concerned with the coordination of reflexes having considerable importance to the animal. In addition, the large diameter of the receptor axons, and their uninterrupted course to the brain (Alexandrowicz, '51; Wiersma, '58) suggest that the receptors are involved in the coordination of rapid reflex responses, since these anatomical characteristics would presumably facilitate rapid transmission of information from the stretch receptors to the brain of the animal.

The most obvious reflex action of the crayfish abdomen is the repetitive flexion which constitutes the escape response (swimming reflex). When alarmed, the animal normally first extends its abdomen ("tail"), flares its uropods, and then strongly flexes the abdomen. A series of such movements cause the animal to move rapidly backwards in the water, away from the source of stimulus. Stimulation at the head end brings into play the medial giant axons, while tactile stimulation of the tail causes discharge of the lateral giants. A single impulse in any one giant axon is normally sufficient to evoke a maximal flexor contraction (Wiersma, '61).

It has been assumed that during the swimming reflex the stretch receptors discharge in response to the strong tail flips as they are known to do when the experimenter passively flexes the tail of the animal. Furthermore, since the RM's were first described, they have been suspected of playing an important role in the coordination of the swimming reflex. Alexandrowicz ('51), for example, suggests that "stimulation of the nerve-cells takes place during stretching of the muscles RM, i.e., when the abdomen is bent by the action of its ventral muscles. At that instant the function of the stimulated nerve-cell of MRO (muscle receptor organ) might be to transmit inhibiting impulses in order to bring about relaxation of the mighty flexor muscles."

In contrast to the proposal that the stretch receptors serve to inhibit flexor discharge, Wiersma et al. ('53) suggest with equal logic that the "impulses from the

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receptor operate to induce reflex contraction of the extensors so that the tail is readied for its next flexion.” A more recent suggestion advanced by Wiersma (’60) is that the discharge of the stretch receptors during the swimming movements may cause reinforcement of the sensory influx that is required to trigger the “firing” of the giant fibers.

It is quite evident that it is not feasible to determine, *a priori*, which of these suggestions for stretch receptor reflex involvement, if any, is correct. In order to arrive at an understanding of the normal function of the crustacean stretch receptor system additional experimentally determined answers are required. When, for example, are the receptor muscles excited to contract so as to increase receptor sensitivity? Are there any direct reflex effects of stretch receptor stimulation in addition to the feedback inhibition resulting from receptor discharge (Eckert, ’61)? Under what circumstances, if any, is the accessory fiber (receptor inhibitor) activated independently of the stretch receptor discharge? Finally, what effect does surgical interference with the stretch receptor system have on the swimming reflex? By combining motion picture analysis of the swimming movements with electrophysiological techniques, answers to these and other questions concerning the normal behavioral role of the stretch receptor system were sought.

METHODS AND MATERIALS

In these experiments, as in the experiments described in the first paper of this series, it was important to examine central and peripheral nervous interactions with a minimum of disturbance to neural connections. The electrophysiological experiments described here were therefore performed with preparations and methods essentially similar to those already described in the previous paper (Eckert, ’61). The saline bath, however, was kept at a higher temperature to facilitate motor activity by the crayfish. A temperature of 15 ± 1°C was found to be suitable, allowing active tail extension, swimming movements, and other normal neuromuscular activity. Such movements can be evoked with some degree of difficulty in the immobilized animal by such stimuli as ru bing of the eyestalks and surroundin areas with a blunt probe, tactile stimul tion of the mouthparts, and tactile stim lation of the ventral surface of the a domen. Some preparations consistentl failed to respond to stimulation, and ha to be discarded.

In addition to electrophysiological investigations of the stretch receptor role i the swimming reflex, it was found usefu to photograph, in “slow motion,” the swimming movements of normal crayfish an those of animals with surgically disconnected receptor muscles (RM’s). Cinematographic records were made at 64 frame per second with a 16 mm motion pictur camera while the experimental anima was stimulated to swim the length of narrow, water-filled lucite channel. I order to facilitate comparisons with th electrophysiological experiments, the wate was maintained at 15 ± 1°C during th behavioral tests. The developed motion picture film was studied, frame by frame with a film editing device.

Because of the number and wide distribution of the abdominal stretch receptors their destruction in the intact animal i difficult to bring about without some bloo loss. To minimize bleeding in those animals used in behavioral experiments, the following method was employed to disconnect all abdominal RM’s. The anima was held under a dissecting microscope with its tail in a flexed position. This allows the investigator to observe, through the semi-transparent dorsal articular membranes of the tail, the superficialis medialis muscle (Eckert, ’61, fig. 1) just anterior to its insertion into the tergum posterior to the joint. Because they lie near the medial edge of this muscle, it was possible to sever the RM’s by means of a fine stainless steel hooked needle. The hook was introduced through a small hole in the articular membrane. Since it did not seem possible to identify, through the articular membrane, the RM’s themselves, it was necessary to sever, along with the RM’s those fibers of the superficial medial muscle near the margin of the muscle. A control experiment was performed, however, in which an equivalent amount of this muscle (not including RM’s) was severed

This animal exhibited a swimming reflex similar to those of unoperated animals.

In those electrophysiological experiments in which destruction of abdominal stretch receptor organs was required, the receptors in all segments were laid bare before the experiment. At the proper moment each receptor was quickly denervated or destroyed without otherwise disturbing the preparation.

In order to differentiate between those tail movements initiated by neuromuscular activity of the animal, and those resulting directly from manipulations by the experimenter, the former are referred to below as "active" movements, while the latter are referred to as "passive."

RESULTS

1. RM contraction during tail extension

During the course of electrophysiological experiments in which the immobilized animal was stimulated to evoke the violent tail flip associated with the swimming reflex, it was seen that prior to tail flexion there was normally a tail extension. This was accompanied by an efferent motor discharge that could be recorded in the dorsal nerve. Branches of this nerve innervate the superficial extensor muscles (Eckert, '61, fig. 1), the contraction of which contributes to tail extension. Florey and Florey ('55) found that most of the fibers innervating the stretch receptor muscles of *Astacus* also branch to the superficial extensor muscles. Therefore, it seemed reasonable that during tail extension the RM's might contract along with the extensor muscles proper.

Do the receptor muscles contract during tail extension? If so, what implications might this have concerning stretch receptor participation during the swimming reflex? In designing an experiment to answer the first of these two questions, it was reasoned that if the RM's could be maintained at constant length under moderate tension, an isometric contraction of the receptor muscle would result in an increase in the receptor discharge rate. The experimental preparation that was used for this purpose was similar to that illustrated in figure 1 of the previous paper in this series. The electrical activity of the dorsal nerve connecting the isolated RM's with the central nervous system was displayed and recorded as previously described (Eckert, '61). The mechanically mounted forceps holding the receptor muscle bundles were so adjusted as to cause a tonic, low-frequency RM_1 discharge. Appropriate tactile stimulation was then applied to the animal to evoke a tail extension. If the receptor muscles are excited during normal tail extension, the receptor output should reflect the additional tension on the receptor dendrites by an increase in frequency. Under these experimental conditions such a response to tail extension never occurred in any of the 10 animals studied. The receptor frequency was not affected by tail extension.

Because of the discrepancy between these negative findings and the histological evidence that the RM's share most of their motor fibers with the superficial extensors, it was felt that perhaps the preparation was at fault. This suspicion was confirmed by experiments with several preparations in which *in situ* RM's were made to contract visibly by electric stimuli to the dorsal nerve. The stimuli were completely ineffectual in eliciting an RM contraction after dorsal nerve branches to the extensor muscles lying between the RM's and the stimulating electrodes were cut. Stimulating at a point beyond the cut branches and closer to the RM's again elicited contractions. It was concluded that conduction in the RM motor neurons is blocked by cutting their branches (innervating extensor muscles), as was done when the RM's were isolated for recording in the preparation described above.

In order to avoid conduction block in the RM motor axons it was apparent that it would be necessary to maintain intact the connections between the dorsal nerve and the extensor muscles. This was subsequently accomplished in the isolated RM preparation by severing, near their insertions, both ends of the superficial extensor muscles associated with the experimental RM pair, without damaging the nervous connections to these muscles. The muscles remained attached to the dorsal nerve while the sensory discharge in the latter was monitored. In other respects, however, the preparation remained identical to the one which repeatedly failed to show any receptor muscle contraction during tail extension.

With conduction block thus eliminated, it was readily seen that the extensor neuron discharges occurring during tail extension were accompanied by an increase in the discharge frequency of the isolated receptor, which has only neural connections with the rest of the animal (see fig. 1). The frequency increase accompanying tail extension under these conditions is apparently due to an isometric contraction of the receptor muscles in response to motor excitation. (It has already been demonstrated by Kuffler ('54) that isometric RM contraction has the same stimulating effect on the stretch receptor dendrites as does passive stretch.)

Although these experiments indicated that the receptor muscles do contract during tail extension, a question remained as to whether this contraction of the receptor muscles *in situ* in the normal animal is accompanied by a receptor discharge. During tail extension the insertions of the RM's are drawn closer to each other by the extensor muscles. This means that the RM contraction during normal tail extension would be an *isotonic* contraction instead of the *isometric* contraction that occurs during the experiments discussed above. It is unlikely that an isotonic contraction would excite the receptor dendrites, which Alexandrowicz ('51, '58) concluded are embedded in a non-contractile region of the receptor muscle bundle. The isotonic contraction should, however, lower the stretch length required for receptor excitation. This reasoning was experimentally confirmed in the following manner. The intact dorsal nerve was exposed by "window" cut in one side of segment By means of a long, flexible recording lea it was possible to monitor, during mov ments of the tail, stretch receptor di charges in the otherwise undisturbed do sal nerve. During active tail extension n receptor discharge was seen to accompan the extension. It can be concluded tha the isotonic RM contraction accompan ing tail extension does not in itself stimu late the receptor.

In these same preparations it was see that during passive (manual) movement of the tail, no discharge occurred when th relaxed tail was brought from the full extended position to a position correspond ing approximately to half of full flexion Only then does RM_1 discharge on furthe flexion, with a maximum discharge at fu flexion. RM_2 discharges only at, or nea full flexion. If, however, the animal i made to actively extend its tail, manua flexion of the tail results in an RM_1 dis charge at a position of far greater exte sion than is the case during an absence o extensor discharge. RM_1 will, in fact, dis charge under these conditions during th initial stages of flexion. During tail exte sion isotonic RM contraction apparentl takes up the "slack" of the receptor orga making it more sensitive to the earl stages of an abdominal flexion.

2. *Inhibition of extensor neuron discharg by stretch receptor output*

One of several possible reflex roles o the stretch receptor system may be a cen tral involvement with the motor neuron responsible for tail extension. It is con

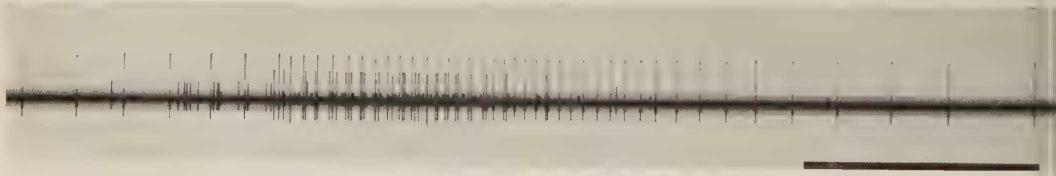

Fig. 1 RM contraction during tail extensor discharge. A stretch receptor pair was isolated and held in a pair of mechanically mounted forceps while their connections with c.n.s. were maintained intact. Moderate stretch applied to the RM's caused a low frequency, tonic RM_1 discharge (largest spikes). During the isometric contraction of the receptor muscle (triggered by extensor fiber impulses seen as the smaller spike sizes in the record) the receptor discharge increased in frequency. As tension decayed following cessation of motor excitation, the receptor frequency characteristically fell below its initial level, presumably in a postexcitatory depression. Time marker indicates 1.0 sec.

ceivable either that stretch receptor discharge during tail flexion plays a role in bringing on subsequent tail extension (Wiersma et al., '53), or that the stretch receptor discharge causes inhibition of extension allowing unopposed tail flexion.

In order to determine whether stretch receptor output acts to inhibit extensor discharge, efferent activity in the dorsal nerve was monitored while the animal was induced to extend its tail. When the tail was in the extended position it was manually flexed. This flexion of the tail caused the extensor discharge to cease (fig. 2A). Upon release of the tail the extensor discharge again appeared and the tail extended, etc. Apparently, the flexion of the tail in some manner inhibits the extensor discharge. Are the stretch receptors responsible for this inhibition of extension by flexion?

By surgical destruction of all the RM's of the tail, it was seen that the stretch receptors are necessary for extensor inhibition during tail flexing experiments. Subsequent to that operation passive tail flexion does not interrupt the extensor discharge as it does when the RM's are intact.

Is the output of a single stretch receptor pair sufficient to inhibit the extensor discharge in a nearby dorsal nerve? Using the semi-isolated stretch receptor preparation (Eckert, '61, fig. 1), it was possible to stretch the isolated RM pair while extensor fiber discharge was monitored in the contralateral dorsal nerve. The stretch receptor discharge and extensor discharge were simultaneously recorded by separate channels. In 6 such preparations, an inhibition of extensor discharge in conjunction with RM discharge was obtained in only one preparation (fig. 2B). Even then, the inhibition was incomplete. It is, therefore, considered likely that extensor inhibition by RM output normally requires a certain amount of spatial summation from the receptors of different segments. An extensor inhibition similar to that resulting from RM stretch can be elicited by

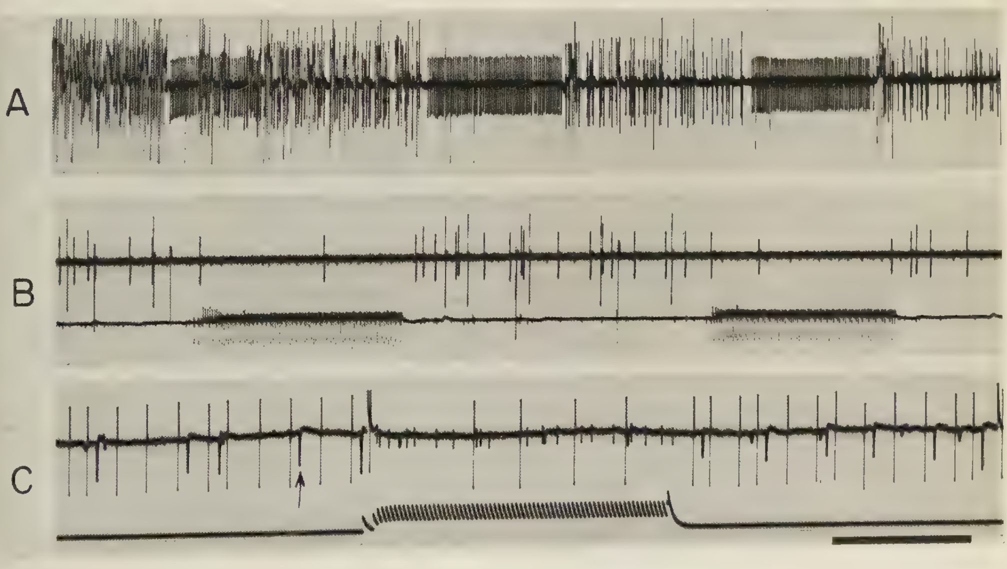

Fig. 2 Inhibition of extensor fiber activity by discharge of the stretch receptors. A, extensor discharge in dorsal nerve (large spikes) inhibited by passive tail flexion. Note feedback activation of accessory fiber (smaller spikes) in response to RM stretch resulting from tail flexion. B, extensor fiber discharge (upper trace) inhibited by contralateral RM_1 output (lower trace). C, efferent fiber impulses (arrow) inhibited by electrical stimulation (50 pulses per second, 3 volts) of the contralateral dorsal nerve (stimulus marker on lower trace). Upper trace also contains RM_1 impulses (largest spikes) from the isolated receptor. Receptor axon stimulation results, in addition to extensor inhibition, in a discharge of the accessory fiber (small spikes) which inhibits the RM_1 train. Time marker indicates 0.5 seconds for all records.

electrical stimulation of the dorsal nerve (fig. 2C). This presumably results from stimulation of the stretch receptor sensory fibers.

Hughes and Wiersma ('60) report the existence of an abdominal interneuron which is activated by RM stimulation in any of the abdominal segments. The posterior branches of the stretch receptor neurons in the abdominal cord apparently synapse with this interneuron in the 5th or 6th (last) abdominal ganglion. Is this neuron, upon being excited by converging RM discharges, inhibiting the extensor neurons of the abdominal ganglia? Apparently it is not, for transection of the cord anterior to the 5th ganglion does not disturb extensor fiber inhibition by tail flexion.

The finding that stretch receptor discharge resulting from passive tail flexion inhibits tail extension suggested that the receptor discharge may have a similar inhibitory effect on extensor discharge during the strong tail flips of the swimming reflex. Receptor discharge in response to the initial flexion might, by extensor inhibition, allow the remainder of flexion to be unopposed by extensor contraction. This suggestion will be considered further on after presentation of additional related data.

3. *Efferent dorsal nerve activity during the swimming reflex*

If it is assumed that the stretch receptor discharge resulting from the initial stages of tail flexion during the swimming reflex inhibits the extensor discharge during the remainder of the flexion, the following question arises: Is the cessation of extensor discharge during active flexion dependent wholly on RM stimulation, or is there also a central provision for timing of the extensor discharge? To answer this question it was necessary to monitor efferent activity in the dorsal nerve during the series of tail extensions and flexions that constitute the swimming reflex. The pattern of efferent discharge was then examined before and after RM destruction.

As in all the electrophysiological experiments described in this paper, the animal was suspended by a thorax clamp, partially immersed in saline. The cut distal end of a second segment dorsal nerve was lifted out of the saline on a pair of hook electrodes, so as to record efferent impulses. Because in this experiment the swimming motions would be performed while the animal was restrained from moving backwards in response to the paddling action of the tail, the water level of the operating chamber was lowered to reduce the resistance that the tail would encounter. It was thought that this would more nearly simulate the resistance that the tail normally encounters.

An occasional series of extensions and flexions could be induced under these conditions by tactile stimuli applied to the animal's head. The associated efferent dorsal nerve discharges were displayed and photographed (fig. 3A). Under these experimental conditions, tail extension always preceeds flexion. The number of fibers discharging is not large—usually about three spike sizes are seen. The fibers attain high discharge frequencies, however, sometimes as high as 200 per second for one spike size. The beginning of flexor contraction can be determined in recordings made with leads so positioned as to cause a deflection of the oscilloscope beam when the flexors are electrically active. Since this large deflection obscures some of the efferent activity, that method of recording was used only for purposes of determining, by comparison, the time relationships of other recordings. The most prominent and reproducible event in recordings of efferent dorsal nerve activity during the swimming reflex is the cessation of extensor neuron discharge during the contraction of the flexor muscles (fig. 3A). For a period of approximately 40 milliseconds the extensor neurons are quiet. This is similar to the time it takes for the fully extended tail to attain a posture of full flexion (see section 4). This extensor inactivation is significant, for the relatively small extensor muscles would most likely suffer damage if they were stretched, while in the excited state, by contraction of the antagonistic flexor muscles.

In the preceding section (2) it was shown that during *passive* tail flexion the discharge from the stretch receptor system was effective in inhibition of extensor

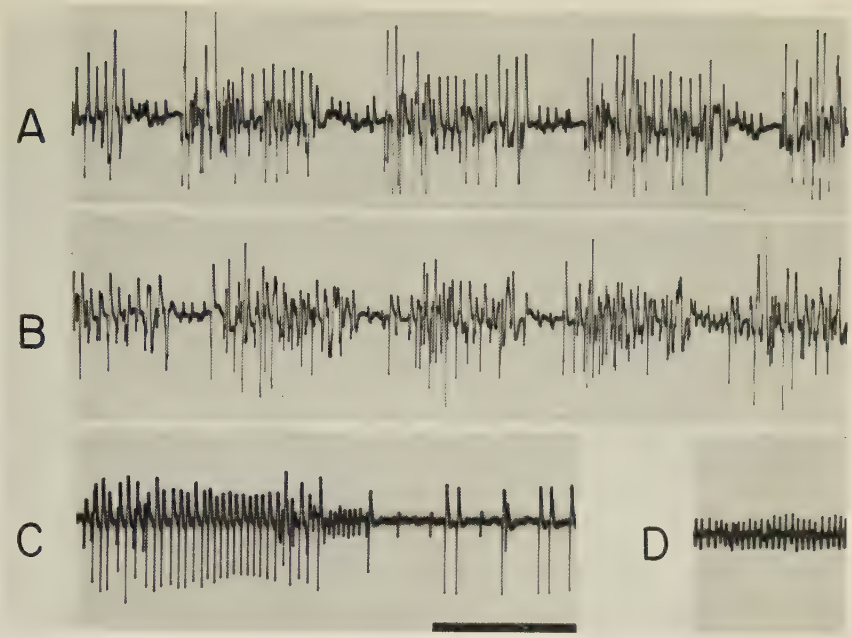

Fig. 3 Efferent dorsal nerve discharges during swimming reflex. A, four cycles of swimming reflex in normal animal. Efferent activity recorded from dorsal nerve. Record begins with discharge of tail extensor neurons (large spikes). At beginning of flexion extensors become silent for a period of about 40 milliseconds before resuming activity for next cycle. Note small spikes to be seen during extensor silence. By comparisons in the same preparations it is seen that these represent impulses in the accessory fiber. B, a similar recording from same preparation as in A after destruction of all abdominal RM's. Extensor muscles of abdominal segment 1 were cut to prevent mechanical transference of tail flexion to RM's of thorax. Differences in state of electrode-nerve relationship resulted in somewhat different spike sizes and shapes in this recording. Note, however, the basic similarity between efferent discharge patterns before and after stretch receptor inactivation. C, a single tail flex cycle (extension, giant axon impulse) in a preparation in which third abdominal roots were cut, interrupting flexor innervation. In the absence of tail flexion the abrupt cessation of extensor discharge accompanied by accessory fiber activity persists in response to giant axon discharge. D, same preparation as in C. Tail passively flexed to evoke accessory fiber discharge for comparison with similar spikes in C recording. Time marker indicates 100 milliseconds for all records.

discharge. For this reason it was suspected that the discharge of these receptors was required during the *active* tail flexion of the swimming reflex in order to inhibit extensor discharge. When all abdominal RM's were destroyed, however, it was found that the extensor neurons stopped "firing" during tail flexion even in the absence of stretch receptor discharge (fig. 3B). This result was completely consistent in several tests conducted on each of the 4 animals having all abdominal RM's destroyed. It was therefore concluded that, in addition to the inhibition by stretch receptor output, there is a centrally determined cessation of extensor discharge coordinated with tail flexion during the swimming reflex.

During the active tail flip, while the extensor neurons are "silent," a train of comparatively small spikes of one size can be seen at a frequency of 150–250 per second (fig. 3). By means of comparisons in the same preparations, it is evident that these spikes are identical with those of the inhibitory "accessory fiber" that was shown (Eckert, '61) to be reflexly activated by stretch receptor discharge. It was reasonable to suppose that this fiber was responding to stretch receptor discharge during active tail flexion, just as it is known to do during passive flexion. If this were true it could be predicted that RM destruction would result in the disappearance of the inhibitor spikes during active tail flexion. To the contrary, figure 3B shows that even after the stretch receptors of the tail are destroyed this fiber continues to discharge during the tail flip. After that operation passive flexion is, of course, no longer effective in causing an inhibitor discharge. It was postulated, therefore, that the accessory fiber is under direct central control in addition to responding in a simple reflex manner to stretch receptor discharge. That is to say, in addition to being reflexly excited by stretch receptor discharge, the accessory fiber is apparently activated by the same central mechanism which is involved in the coordination of the swimming reflex.

In order to be certain that the accessory fiber discharge was entirely central in origin, the flexor muscles were denervated by severing all the abdominal cord third roots. Since this operation prevents active tail flexion, it was reasoned that it would prevent sensory influx (from joint hairs, etc.) during the discharges that would normally evoke a series of tail flips. Figure 3C shows a dorsal nerve discharge of a single cycle of the swimming reflex—one extension and flexion—after the third roots were cut. (The "flexion" in this case is, of course, represented only by neural events, since the flexors are denervated by the operation.) Note the discharge of the smaller fiber following the abrupt cessation of the extensor discharge. That these spikes represent activity in the inhibitory accessory fiber can readily be seen by comparing them with those of figure 3D, which are accessory fiber impulses elicited in the same preparation by a passive tail flexion. This experiment leaves little doubt that in addition to being reflexly excited by stretch receptor discharge, the accessory fiber is excited by the same central mechanism that effects the neuromuscular events of the swimming reflex.

4. *Cinematographic analysis of the swimming reflex before and after RM destruction*

In conjunction with the foregoing electrophysiological experiments, it was felt that some information concerning the role of the stretch receptor system in the swimming reflex could be gained by behavioral observations. What effect on the coordination of the swimming movements would RM destruction have? If these organs are of great importance to such coordination, their removal should result in an obvious disruption of the reflex.

Because of the rapidity of the swimming movements, it was found necessary to analyze them by means of slow motion cinematography. At 64 frames per second the individual frames of motion picture film were exposed approximately 15.6 milliseconds apart while the animal was prodded into swimming across the field of the camera lens.

The most constant portion of the swimming cycle, with respect to duration, is the flexion of the tail. From the position of extension the position of greatest flexion was normally attained in three frames or in about 45 milliseconds. The other portions of the swimming cycle, however, were subject to considerable variation in duration. This variation exists not only from one individual to another, but occurs from one cycle to another in a series of tail flips. Individuals differ also in the position to which the tail is extended during each cycle, some animals exhibiting more extreme extensions than do other animals.

After the normal animals were photographed, they were chilled in ice water before their abdominal RM's were disconnected at their posterior insertions. (It can be argued that this operation is incomplete, leaving the thoracic RM's intact. This is true. In the foregoing electrophysiological experiments this problem was overcome by severing the extensor muscles of abdominal segment 1. This had the effect of disconnecting the thoracic receptors from the movements of the tail. This operation was, of course, not feasible in the case of the behavioral experiments. However, it seems doubtful that the presence of the thoracic receptors could compensate to a large degree for the destruction of all the abdominal receptor organs.) After about 30 minutes of recovery time, the operated crayfish were stimulated into swimming while a photographic record was again made.

Comparison of the swimming movements of operated animals with those of normal animals revealed very little obvious difference. It appears that the operated animals may extend their tails to a slightly lesser extent than do the normal animals. This difference is, however, not at all clear cut, and is no greater than differences in the degree of tail extension from one normal animal to another. Upon close examination it also appears that after the operation the onset of tail extension occurs somewhat sooner after full tail flexion than it does in the normal animal. Considerable normal variability in the time of the onset of tail extension tends to obscure this effect of the operation, however. In general, the behavioral observations indicate that the animal swims nearly as efficiently without its abdominal RM's as it does with them. These findings are supported by the neurophysiological observations which indicate that the efferent discharge to the extensor muscles and stretch receptors is altered very little by RM destruction (fig. 3).

DISCUSSION

What role do the stretch receptors play in the reflex activities of the crayfish nervous system? This question might have been answered with some degree of confidence had this investigation been concerned only with those experiments described in sections one and two, i.e., the experiments concerning RM contraction during tail extension, and inhibition of extensor neuron discharge by stretch receptor output. The following reasoning might have been possible to explain RM function in the swimming reflex: During the tail extension that precedes flexion the stretch receptor muscles contract isotonically, taking up the slack of the RM's so that during the initial stages of the ensuing flexion the receptors respond with an intense discharge. The stretch receptor discharge, upon reaching abdominal ganglia, inhibits further extensor discharge, allowing the remainder of flexion to proceed unopposed by the extensors. In addition to centrally inhibiting extensor discharge, the RM discharge then inhibits itself partially by reflex excitation of the accessory fibers (Eckert, '61), releasing the inhibition of extensor neurons. The discharging extensors then bring about a new tail extension, and the next giant axon impulse sets off another tail flex cycle.

This explanation does not seem entirely satisfactory in the light of results from the subsequent experiments. First it was seen that the extensor fibers become silent during the flexion of the tail even though the RM's were destroyed. The cessation of extensor discharge occurred even when cutting of the third ganglionic roots prevented tail flexion in response to giant axon impulses, ruling out other sensory input as the cause for extensor "cut off" during the active tail flexion. It had to be concluded that there was a central timing of extensor discharge somehow associated with the discharge of the giant fiber system. Second, the train of accessory fiber impulses during the extensor silence was found to persist after stretch receptor destruction. It was, therefore, not activated during the tail flip in the same feedback manner as it is when the tail is passively flexed. The accessory fiber, it was concluded, was being made to discharge by some central elements without the necessity of peripheral intervention.

What components of the c.n.s. are responsible for the repetitive firing of the accessory fiber during the tail flip? Wiersma ('52) described repetitive postganglionic discharges by a second root fiber in response to single giant axon impulses in the crayfish. Each giant axon impulse was followed by a short series (usually less than 10) of small second root spikes having a frequency of between 200 and 300 per second. The after discharge was delayed from 5 to 10 milliseconds after the prefiber impulse. The same fiber was present in the second roots of all the abdominal ganglia. Several correlations indicate that this afterdischarging second root fiber is identical with the accessory fiber: (1) Both are present in the same abdominal roots. (2) The discharges of both are associated with giant axon impulses. (3) The discharge frequencies and train lengths of both are similar. This evidence indicates that in addition to triggering flexor contraction the giant fiber impulse inhibits, via the accessory fibers, the discharge of stretch receptors. It may also directly, or via interneurons, inhibit the discharge of the extensor neurons.

The high frequency discharge of the accessory fiber in response to a giant fiber impulse implies that the stretch receptor system is kept silent during the entire swimming reflex. This, however, may not be entirely true. Examination of figure 3A indicates that the accessory fiber discharge stops before the beginning of tail extension. This should allow the uninhibited receptors to discharge at their point of maximum stimulation, while the abdomen is fully flexed. (Unfortunately, the large flexor muscle potentials have made it impossible to record this receptor discharge from the intact dorsal nerve during the tail flip.) After a certain latent period this sensory discharge, by means of feedback inhibition, would be sharply reduced. Such a sequence of excitatory and inhibitor events would have the effect of producing a sharp burst of stretch receptor impulses when the tail is maximally flexed. This afferent burst from the stretch receptors in addition to exciting the accessory fiber and inhibiting the extensors, arrives at the telson ganglion and the brain along uninterrupted branches of the receptor axons.

What does this burst of sensory information do at these two extremities of the nervous system? It is interesting to note that the brain is the origin of the impulses carried by the medial giant axons, while the telson ganglion is the origin of the impulses carried by the lateral giants. Wiersma ('60) was led by this observation to suggest that the stretch receptor serves to augment the exteroceptive input, and thus leads to repetitive discharge of the giant axons, which are relatively hard to excite. The present experiments, however, do not support this attractive hypothesis, for removal of the RM's does not seem to change the sensory threshold of the swimming reflex. Nor does it seem to affect the frequency of the swimming movements. A comparison of figures 3A and 3B reveals that the frequency of tail flips was higher in the record made after destruction of the RM's than in the record made before the operation. This recorded difference in postoperative tail flip frequency is coincidental, for it is evident from other experiments that the frequency of tail flips varies considerably within a normal animal, and is not consistently increased or decreased by RM destruction. These same records, however, do suggest another, seemingly minor, function of the stretch receptor system. After receptor destruction (fig. 3B) the extensors become active somewhat sooner after flexion than in the normal discharge pattern (fig. 3A). This might be explained by the absence, in the operated animal, of stretch receptor discharge (following cessation of the accessory fiber impulse train) at full tail flexion. Normally, this discharge must act to inhibit the initial discharge of extensor neurons following tail flexion, just as stretch receptor discharge during passive tail flexion inhibits the extensor neurons. Subsequent feedback inhibition via the accessory fiber presumably lowers the stretch receptor discharge frequency enough to allow the resumption of tail extension. This hypothesis is consistent with the observed effect of RM destruction upon swimming behavior. Instead of slowing the frequency of tail flips in an appropriately stimulated animal, RM destruction leaves the frequency the same. The interval between tail flexion and tail extension is definitely not increased—the two shortest of these intervals measured in cinematographic records (15 milliseconds, or less) both came from animals with the abdominal RM's destroyed. Hence, the role of the stretch receptors during the swimming reflex appears to be limited to the effects of their discharge at the moment of full flexion. The receptor discharge at that moment would be expected to delay the onset of extensor contraction, as actually shown in figure 3.

This role of the stretch receptors in the swimming reflex, while more limited than the roles previously proposed (Alexandrowicz, '51; Wiersma et al., '53 and Wiersma, '60), might still be of importance to the animal. By facilitating coordination of flexion and extension, the propelling efficiency of the tail movements might be improved. Superficially, however, it would seem that the delay in onset of extensor contraction might be more simply accomplished via the central mechanism which is already known to be concerned with the timing of the extensor discharge.

There are, of course, other less obvious possible functions of the stretch receptors. One is that they may play a larger role in the swimming reflexes of younger animals. The RM's might, for example, be used for the coordination of the swimming movements until the young central nervous system "learns" the reflexes (e.g., by conditioning) sufficiently well so that the swimming reflex is no longer dependent on the sensory influx from the stretch receptors. This particular hypothesis received support from the observation that during tail flips the central nervous system does perform those functions (inhibition of extensors, excitation of accessory fibers) which the stretch receptors readily perform during passive flexion.

As a result of the present findings, the following general observations can be made concerning the sensory physiology of the stretch receptor system: (1) During passive flexion of the crayfish tail (and thus presumably also during slow active flexion) there is a fairly direct relationship between the degree of abdominal flexion and the frequency of RM_1 discharge. This sensory discharge is subject to a certain amount of modulation by inhibitory feedback and by RM contraction, but is effective in presenting the c.n.s. with continuously graded tonic proprioceptive information. (2) During the maximal tail flexion triggered by the giant axons the stretch receptors are kept silent by centrally originating accessory fiber impulse trains until full flexion is achieved. At full flexion, the receptors, then released from inhibition, are free to respond with a brief high frequency discharge before the subsequent extension takes place.

The stretch receptor system can, therefore, be said to exhibit two kinds of responses during two kinds of tail flexion. One response consists of variable frequencies of impulses related to the degree of tail flexion. The other response consists of a brief high frequency discharge occurring only at the moment of full tail flexion. The presence of a double receptor function in certain crustacean sensory systems is not unknown. Cohen ('55) reports that the type II position receptor of the lobster statocyst signals both static position and angular displacement direction. The dual sensory function of this mechanoreceptor is apparently a property of the receptor itself, and does not depend on external control. The stretch receptor system, however, responds in a complex manner primarily because of central modulation of receptor output. Moreover, the central nervous system of the crayfish determines the type of stretch receptor response in keeping with the kind of flexor motor activity which is centrally evoked.

SUMMARY

1. The influence of the stretch receptor system on the coordination and timing of the crayfish swimming reflex was investigated by means of electrophysiological and cinematographic techniques.

2. The extensor neuron discharge during tail extension was shown to result in contraction of the stretch receptor muscles. In the experimental situation the isometric RM contraction gave rise to an increase in receptor output frequency. *In situ* the isotonic RM contraction takes up the slack of the receptor muscle during tail extension.

3. Stretch receptor discharge was shown to be effective in centrally inhibiting the discharge of extensor neurons. Spatial summation is required for effective inhibition.

4. Recordings from the dorsal nerve (innervating the RM's and extensor muscles) during the repetitive tail flips of the swimming reflex indicate that during tail flexion the extensor neurons are silent. Ablation experiments showed that the stretch receptor system is not required for inhibition of the extensors during the swimming reflex.

5. The same recordings indicate that, during the extensor fiber silence, the accessory fiber discharges at high frequency with or without the integrity of the stretch

receptor system. The accessory fiber is apparently discharging directly in response to giant axon impulses. Because of this inhibitory fiber discharge, the stretch receptors are inhibited during most of the tail flexion, and evidently discharge in an abrupt burst only when the accessory fiber stops firing, at full flexion.

6. Although cinematographic studies revealed no gross deficiencies in the swimming behavior of animals in which the RM's had been cut, close examination did reveal that the shortest intervals between full flexion and the beginning of tail extension occurred in those animals whose RM's had been destroyed. This observation is in agreement with the electrophysiological observation that after RM destruction the extensor neurons begin firing sooner after full tail flexion than in the normal animal.

7. Because of centrally originating inhibition of the receptor output the stretch receptor system exhibits two kinds of responses to tail flexion. During slow flexion the receptors respond to different degrees of tail flexion with a graded discharge frequency. However, during the violent tail flip triggered by the giant axons, the receptors, because of centrally originating inhibitory fiber activity, are free to discharge only at the fully flexed tail position with a short, high frequency train of impulses.

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A Contribution to the Studies on Changes of Energy Metabolism During Postnatal Development

I. DEVELOPMENT OF MECHANISMS OF BODY TEMPERATURE REGULATION IN RATS

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Studies on changes of metabolism during the development of animals are difficult to make because there is no simple way to compare the findings on newborn animals with those of adults. According to Brody ('45) such comparisons are of limited value for three reasons: (1) Infant animals are not completely homeothermic, making it difficult to establish basic conditions for them; (2) the relative amount of muscle tissue, in which most of the heat is generated, varies with growth; (3) during growth the shape of the body changes, so that no simple relation between body weight and surface area can be established; furthermore, the thermal conductivity of the skin changes with the increase in body insulation (fur and epidermis).

When an attempt is made to express the metabolic rate as the amount of oxygen consumed per unit of surface area and per unit of time, or, as suggested by Kleiber ('47), in proportion to $W^{3/4}$ for infant and adult animals, one finds that the values thus obtained differ in biological significance. The present report is concerned with the problems which arise in such investigations.

Gulick ('37) was the first to show that rats up to 18 days of age are unable to maintain a constant body temperature. Brody ('43); Hill ('47); and Buchanan and Hill ('47) studied young animals at 15°C and at 2 to 4°C. Under these environmental conditions the metabolism of one-day-old rats, which have a relatively large and naked external surface, would have to be "unbelievably" high in order to allow for typical mammalian body temperature. On the other hand, even at higher environmental temperatures, such as 20 to 30°C, these animals are incapable of thermoregulation. In this regard inadequate heat production and/or heat conservation may be involved.

Although heat production in infant rats at different temperatures was studied by Antoschkina ('39) and Fairfield ('48) in terms of oxygen consumption, no experimental data are available regarding the influence of temperature upon the cutaneous circulation of such animals—a problem of considerable importance in heat conservation and dissipation. Data on this aspect of the problem are included in the present study.

MATERIALS AND METHODS

The experiments were carried out on 99 albino rats of the Nelson strain (Carworth Farms). Pregnant females were housed in individual cages with purina chow and water provided ad libitum. The temperature in the animal room was kept at approximately 26.5°C. Only members of the larger litters (5 to 13 animals per litter) were used. The experimental groups consisted of both males and females of the same age and each from a different litter. The temperature of the animals, as well as of the environment, was determined with a thermistor telethermometer (Yellow Spring Instrument Co.). Body temperature of the youngest animals was taken with a subcutaneous probe, and of older animals (16, 18, and 20 days) with a rectal probe. Skin temperature was meas-

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ured with a skin probe on the left side in the middle of the body. In older animals, the body temperature was determined with a rectal probe, and in addition a skin probe was placed on the surface of the hairs and a subcutaneous probe under the hairs to determine the temperature of the skin itself in the same body region.

Prior to the experiments the animals were taken out of their nests and kept for at least 40 minutes at room temperature (approximately 26°C). They were then taped across the legs to a small board allowing for a nearly normal body posture and placed in a thermostatic chamber, in which the temperature was kept at 30 or 35°C for about one hour and then lowered to 25°C. In some of the experiments the animals were initially kept at low temperature (18.5°C) and then transferred into an environment of 25°C. These particular temperatures were selected because they are close to so-called neutral temperature, which for young adult rats is 30 to 31°C (Ware, Hill and Schulz, '47). During the exposure to high temperatures the thermoregulator operated a fan with heater every few minutes, so that the air was periodically but not constantly turbulent; during the cooling period only natural convection was utilized.

The relative humidity in the chamber was about 40%, and small variations probably did not affect the skin temperature to any measurable extent. This is supported by the data obtained on man by Roth and Morton ('40) who found that at temperatures close to neutral (23–28°C) changes in relative humidity of from 30 to 65% had little effect on the skin temperature.

RESULTS

The relationship between changes of environmental temperature and body and skin temperature is shown in figures 1 to 5, which are representative protocols of single experiments. Figures 1 and 2 show the changes of body and skin temperatures in one- and 12-day-old rats respectively, when they were exposed first to 30°C and then to 25°C. Figures 3 and 4 deal with similar experiments performed on one pair of 20-day-old siblings when one of them was shaved. Figure 5 shows changes of body and skin temperatures in a one-day-old rat when environmental temperature was intially lowered to 18.5°C and then raised to 23°C.

Environmental temperature was found to affect considerably both body and skin temperature of young animals, especially below 8 days of age. When about 10 to 15 minutes had elapsed after exposure to a given temperature, a steady state was established in which the difference between body and air temperature remained constant. It is obvious that body temperature of infant rats changes with environmental temperature. These changes are

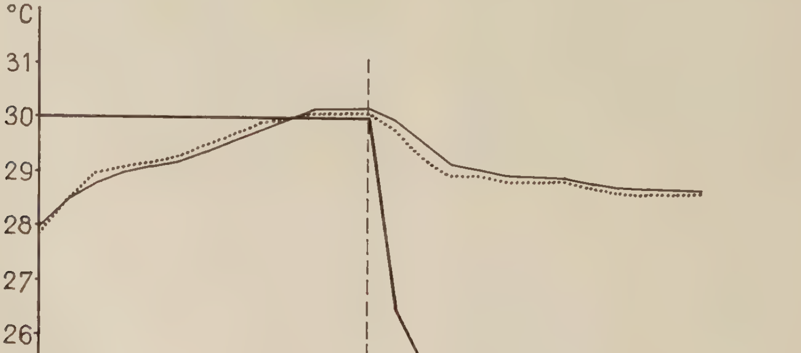


Fig. 1 The change of body (rectal) and skin temperatures in one-day-old rats exposed to 30°C and then to 25°C. Lighter line, body temperature; dotted line, skin temperature; heavier line, air temperature.

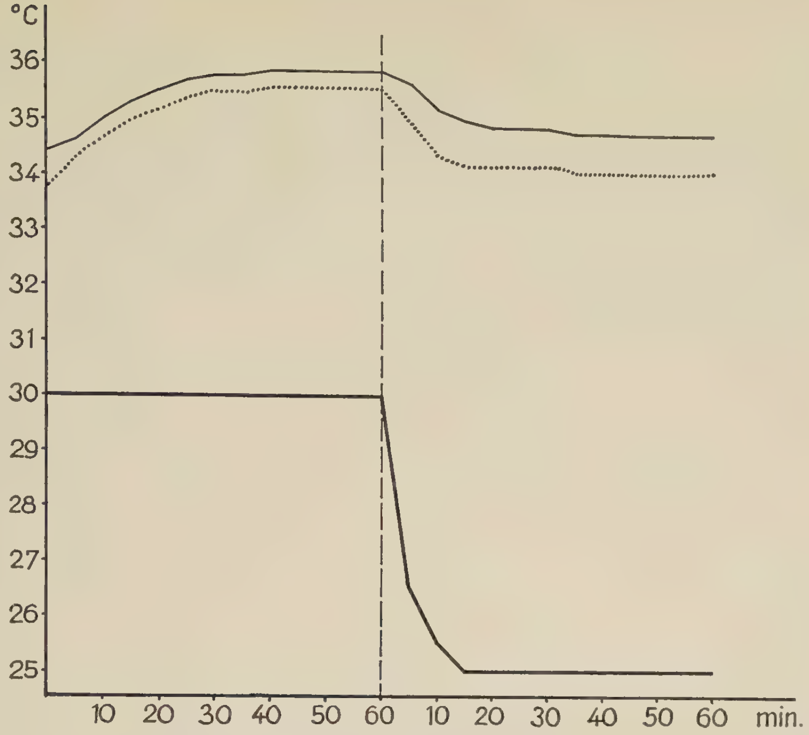


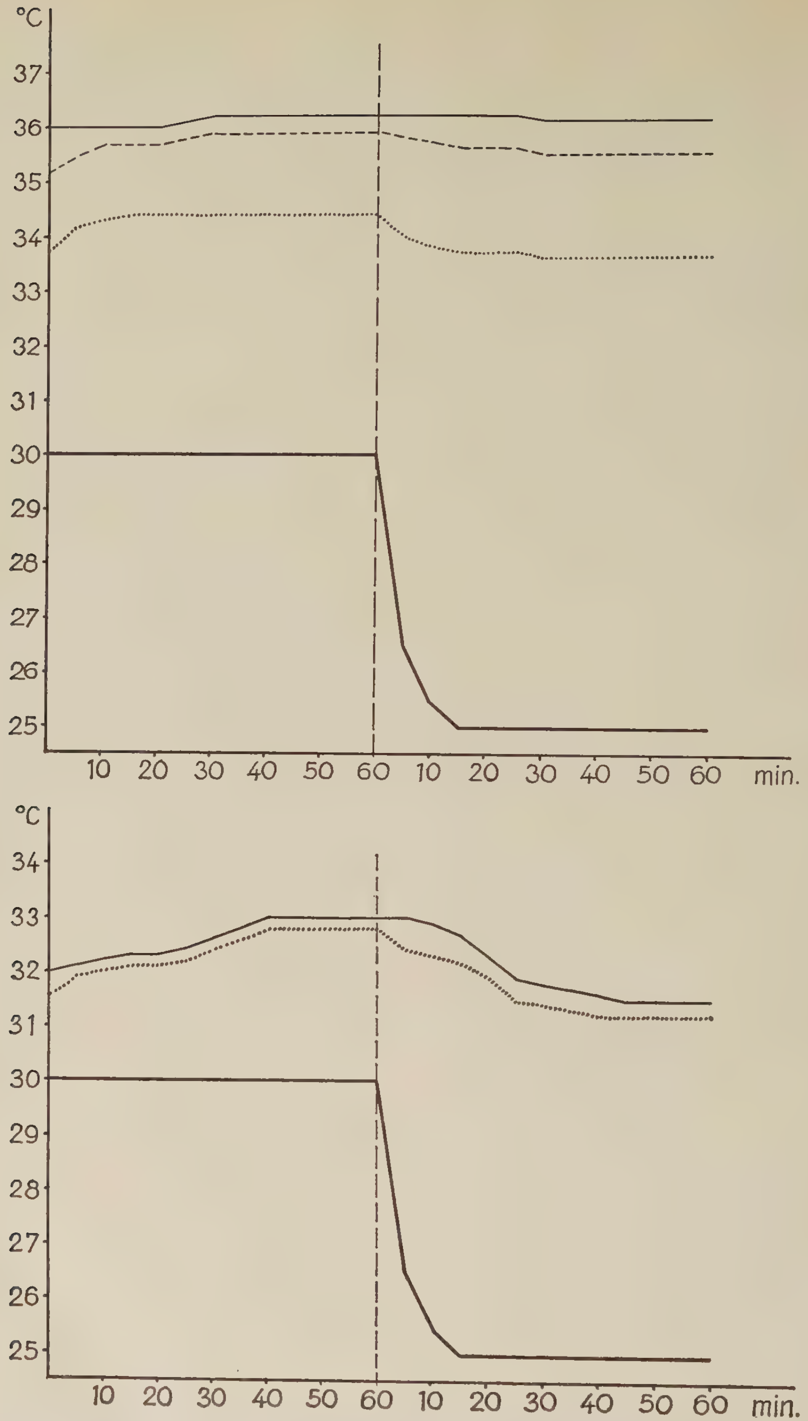
Fig. 2 The change of body (rectal) and skin temperatures in 12-day-old rats exposed to 30°C and then to 25°C. Lighter line, body temperature; dotted line, skin temperature; heavier line, air temperature.

of greater magnitude in one-day-old rats (figs. 1 and 5) than in the older ones (figs. 2 and 3). In 20-day-old rats there was almost no change of body temperature with environmental changes from 25°C to 35°C. When 20-day-old rats were shaved, however, changes in body temperatures were much greater than in unshaved ones. This was demonstrated on 4 pairs of siblings. Figures 3 and 4 illustrate the results on one such pair.

Table 1 lists the results of the experiments in which the animals were initially exposed to 30°C and then to 25°C. The differences between body and skin temperature at two steady states are listed in the last two columns on the right.

In one-day-old animals this difference was 0.1°C during the steady state at either 30°C or 25°C. During the chilling period, however, and until the second steady state was established, a greater difference (0.2 to 0.3°C) was usually noted. On the other hand, when the steady state was reached at 35°C, the skin and body temperature were frequently equal. In older animals, as one might expect, the difference between skin and body temperature was greater. It was also greater in the steady state at 25°C than at 30°C. This difference in older rats (12 days and over) is probably due not only to vascular shunting but to the increased insulation afforded by growing fur.

Eruption of the first hairs in rats occurs on the second day of postnatal life (Fraser, '28; Butcher, '34). Hair as insulating cover is relatively unimportant even in 88-day-old rats. In older animals, however, such insulation has considerable influence on the relative body and skin temperature, as can be seen by comparing the data obtained on 16-, 18-, and 20-day-old rats when the skin temperature was measured on fur (table 1) and under the hair (table 2). With the latter method not only are the differences between body and skin temperature much less, but a much smaller individual variation exists. The large individual variations which were



Figs. 3 and 4 The changes of body (rectal) and skin temperature of 20-day-old siblings exposed to 25°C and then to 30°C. On figure 3, unshaved; on figure 4, shaved. Lighter line, body temperature; dotted line, skin temperature measured on hair; dashed line, skin temperature measured under hair; heavier line, air temperature.

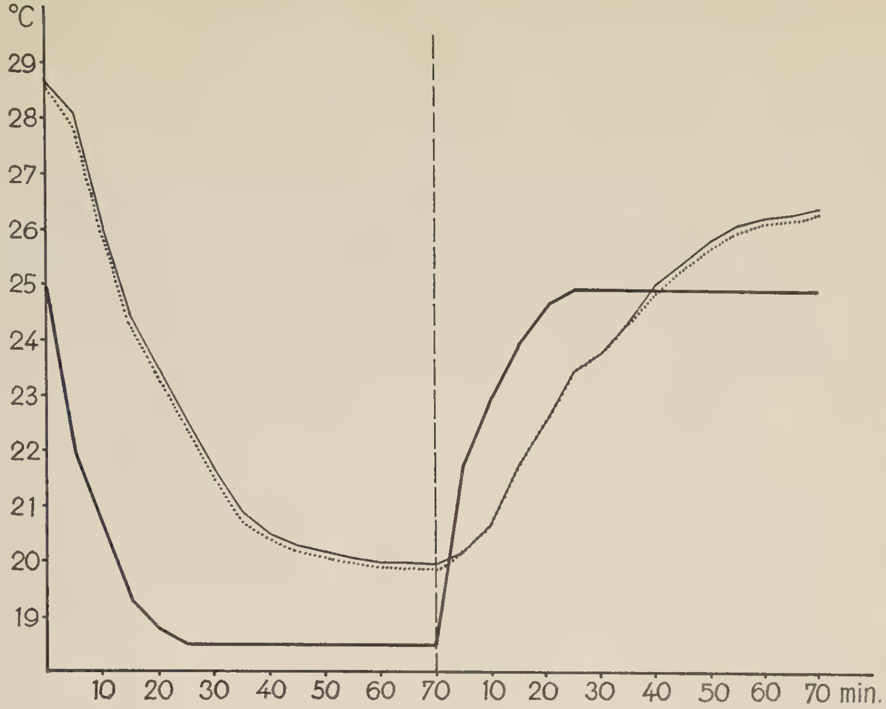


Fig. 5 The changes of body and skin temperature in one-day-old rat when environmental temperature was initially lowered to 19.5°C and then raised to 23°C. Lighter line, body temperature; dotted line, skin temperature; heavier line, air temperature.

noted in temperatures measured on hair surface are in part due to experimental error. The considerable difference in length of hairs on rats of the same age also influenced such temperature readings.

The relative body and skin temperatures at different environmental temperatures provide indirect information about the changes in blood circulation of the skin under the influence of thermal stimuli. In the youngest, hairless rats it was possible to observe these changes directly. The color of the skin of rats over 4 days of age was much lighter at 25°C than at 30°C. On the other hand, in one-day-old rats the same pink color of the skin was observed at either temperature. Local cooling of the skin with the help of a test tube filled with ice had no apparent vasoconstrictor effect.

Table 1 shows that during the steady state at 30°C the body temperature in one-day-old rats was higher on the average by only 0.4°C than that of the environment, and by 6.3°C in 20-day-old rats. During the steady state at 25°C these differences were on the average 3.5°C and 11.3°C for one- and 20-day-old rats respectively. These differences increase with the age of the animals. It is particularly interesting that during the steady state the difference between body and environmental temperature was higher during the exposure to 25°C than to 30°C. During the steady state at 35°C in one-day-old rats the body temperature was either lower or at most equal to that of the environment; on an average it was lower by 0.8°C. Under the same condition in 3-, 8-, and 15-day-old rats the body temperature was uniformly higher than that of the environment on an average of 0.3, 0.4, and 1.1°C respectively.

Marked deviations from the average values (table 1) were encountered especially in the case of the youngest animals. This may be due to the fact that neither age nor weight are adequately correlated to the development of the thermoregulatory mechanisms.

It was found that in a particular age group, the larger animals regulated their

TABLE 1

Experimental data obtained on rats of different age

Age	No. of expts.	Weight	Body temp. at the beginning	Time of heating to steady state	Body temp. during steady state at 30°C	Time of chilling to steady state	Body temp. during steady state at 25°C	Difference between body and environmental temperature (°C)			Difference between body and skin temperature (°C)	
								At beginning	During steady state at 30°C	During steady state at 25°C	During steady state at 30°C	During steady state at 25°C
days		*gm*	*°C*	*min.*	*°C*	*min.*	*°C*					
1	9	5–8	28.4 (26.2–30.1)	46 (35–60)	30.4 (29.6–31.7)	40 (25–50)	28.5 (27.7–29.3)	3.4 (1.2–5.1)	0.4 (−0.4–+1.7)	3.5 (2.7–4.3)	0.1	0.1
4	10	9–12	29.6 (27.3–31.2)	45 (25–60)	31.7 (31.0–32.5)	38 (25–45)	29.7 (27.9–31.5)	4.6 (2.3–6.2)	1.7 (1.0–2.5)	4.7 (2.9–6.5)	0.11 (0.1–0.2)	0.17 (0.1–0.3)
8	9	15.5–21	30.5 (29.5–31.2)	44 (15–55)	31.6 (30.2–33.0)	36 (10–45)	30.2 (27.8–31.1)	5.5 (4.5–6.2)	1.6 (0.2–3.3)	5.2 (2.8–6.1)	0.23 (0.1–0.6)	0.45 (0.3–0.7)
12	8	19–30	33.5 (30.5–35.0)	20 (5–40)	33.7 (32.0–34.9)	35 (15–40)	32.5 (30.4–34.2)	8.5 (5.5–10.0)	3.7 (2.0–4.9)	7.5 (5.4–9.2)	0.6 (0.2–0.9)	1.1 (0.4–1.6)
16	9	25–40	34.2 (33.8–36.6)	16 (5–25)	35.4 (34.8–36.9)	25 (15–35)	35.0 (34.0–36.2)	9.2 (8.8–9.6)	5.4 (4.8–6.9)	10.0 (9.0–11.2)	0.93 (0.6–1.4)	1.54 (1.0–1.9)
18	9	28–39	35.6 (35.2–36.0)	13 (5–30)	35.7 (35.2–36.0)	29 (20–35)	35.2 (34.8–35.8)	10.6 (10.2–11.0)	5.7 (5.2–6.0)	10.2 (9.8–10.8)	1.0 (0.6–1.5)	1.40 (0.9–1.8)
20[1]	11	33–57	36.5 (36.0–36.8)	18 (5–25)	36.5 (35.2–37.2)	19 (10–30)	36.6 (35.9–37.0)	11.5 (11.0–12.8)	6.5 (5.2–7.2)	11.3 (10.9–12.0)	1.86 (1.1–2.5)	2.46 (1.3–3.1)
20[2]	4	32–39	34.9 (34.2–35.5)	38 (20–45)	36.0 (35.8–36.3)	39 (35–40)	35.0 (34.8–35.2)	9.9 (9.2–10.5)	6.0 (5.8–6.3)	10.0 (9.8–10.5)	0.4 (0.3–0.6)	0.75 (0.7–0.9)

[1] Unshaved.

[2] Shaved.

TABLE 2

Differences between body and skin temperature

Age	No. of rats	Weight	Difference between body and skin temp.[1] during steady state at 30°C	Difference between body and skin temp. during steady state at 25°C
days		*gm*	°C	°C
16	4	25–36	0.27 (0.2–0.3)	0.50 (0.4–0.7)
18	5	28–38	0.28 (0.2–0.4)	0.58 (0.4–0.8)
20	9	33–45	0.29 (0.2–0.4)	0.60 (0.4–0.8)

[1] Skin temperature was measured under the hair.

body temperature better than smaller ones. However, older animals of smaller size showed better thermoregulation than younger ones of equivalent size. The latter observation is in agreement with the findings of Hill ('47).

DISCUSSION

A direct application of the conventional thermal circulatory index (Burton, '34) was not possible, since our experimental rats did not behave as homeothermic animals. The fact, however, that in one-day-old rats body and skin temperature remained nearly equal, while the environmental temperature changed from 25 to 30°C, clearly indicates that thermal stimuli do not produce constriction or dilation of cutaneous vessels in these animals. This conclusion is further supported by the fact that local cooling of the skin does not produce any changes in the color of the cooled region. Apparently, one-day-old rats have no vasomotor mechanism for heat conservation and dissipation. This mechanism develops gradually with age, as evidenced by the findings that after 8 days of age thermal conductivity of the skin decreases and is greater in the steady state at 30°C and smaller at 25°C.

During chilling, a greater relative difference existed between skin and body temperature. One may presume, therefore, that the decreased cutaneous circulation protects the organism against cold mainly when a rapid drop in environmental temperature takes place. Probably, as the cold stimulus continues, heat production rises, making it possible to maintain a definite difference between body and air temperature, even in the absence of strong vasoconstriction.

Shaved rats showed a greater difference between body and skin temperature than controls. This suggests that there is an interdependence between vasomotor mechanisms responsible for heat conservation and the physical factor of body insulation. Actually, in shaved 20-day-old rats, in spite of stronger vasoconstriction, the body temperature was more variable than in unshaved ones, showing the importance of the hair in body temperature regulation.

Several investigators have reported that young or newborn rats are essentially poikilothermic (Gulick, '37; Brody, '43; Hill, '47). In our experiments, at environmental temperatures of 25 and 30°C, the temperature of the body, even in one-day-old rats, was higher than that of the environment. Moreover, this difference was greater at 25°C than at 30°C. On the other hand, at 35°C the body temperature in one-day-old rats at most was equal to that of the environment. In this sense, such animals behaved like homeotherms, but since the body temperature of infant rats was also variable, they were truly neither homeothermic nor poikilothermic. In this respect, they were similar to so-called heterothermic animals, as are some primitive mammals, including *Echidna*, *Ornithorhyncus*, some *Marsupialia*, and *Chiroptera* (Prosser et al., '50). In heterothermic animals the body temperature at normal activity is usually higher than that of the environment, but much lower than in higher mammals. It appears, therefore, that rats in early postnatal life pass, in respect to temperature regulation, through the phylogenetic stage reached by more primitive mammals.

One may conclude from the above that it is possible to establish for infant rats a median or “quasi normal” body temperature. It is apparent that other authors failed to notice this fact since they selected much lower environmental temperatures. According to Hill (’47), the normal body temperature in rats increases progressively from 36.5° at 18 days to 38.2° at 30 days or more. Our results show a similar trend in rats younger than those investigated by Hill. In rats less than 18 days old the “quasi normal” body temperature is even lower, namely at 1, 4, 8, 12, and 16 days it is about 29, 30, 31, and 35°C respectively.

The absence of a vasomotor mechanism for heat conservation in infant rats is interesting in view of the fact that (1) the body temperature of these animals is higher than that of the environment when the latter is 25°C, and (2) at 30°C the difference between body and environmental temperature is smaller than at 25°C. A plausible explanation of the above would be that the thermal stimuli coming from the environment cause changes in heat production which are more evident at lower temperatures.

It would be useful at this point to recall some of the general principles involved in the relationship between the metabolism of homeothermic animals and environmental temperature. Thermal neutrality represents the range of environmental temperatures at which heat loss from the body is equal to the minimum heat production. This thermal neutrality is usually about 7 to 10°C below the rectal temperature and is different for the various species of animals (Brody, ’45). Two critical temperatures, a low and a high one, are also usually recognized. Within a certain range of temperatures below the low critical one, the animal exhibits extra heat production. Further decrease in environmental temperature leads to so-called low lethal temperatures, at which both the heat production and the body temperature decrease. Above the high critical temperature the body temperature rises and the metabolism increases according to Vant Hoff’s Law; near the high lethal temperature, however, metabolism again decreases.

The influence of temperature upon oxygen consumption, i.e., heat production, of young rats was investigated by Antoschkina (’39) and Fairfield (’48). Accord to Antoschkina, oxygen consumption in 10-day-old rats (31 days post-conception), was highest when colonic temperature of the animal was 30°C, namely 65 ml/kg/minute. When the body temperature was either below or above 30°C, oxygen consumption decreased. Thus, at a body temperature of 33°C it was only about 30 ml/kg/minute. In older animals the decrease in oxygen consumption occurred at different body temperatures, namely at 25, 36, and 38°C for 17-, 26-, and 30-day-old rats respectively.

Fairfield (’48) studied the respiratory metabolism of rats from birth to 17 days of age. He found at the environmental temperature of 35°C and at equal body temperature that oxygen consumption remained quite constant with age and ranged from 21 to 36 mm^3/gm/minute. When the animals were transferred to 20°C, those over three days of age showed an immediate increase in oxygen consumption. In 15-day-old rats, this increase occasionally reached 170% during the first 15 minutes. The metabolism remained elevated for about 30 minutes and then during the ensuing one to 4 hours returned to the original level. When the animals were transferred to 10°C, only those over 8 days of age showed an increase in oxygen consumption, whereas in the younger ones a decrease was immediately noted. Similarly, Cassin and Otis (’60) also found in infant mice a greater oxygen consumption at 25 and 30°C than at 10°C.

Since “quasi” normal body temperatures can be determined in infant rats, it is also possible to establish the characteristic environmental temperature discussed above. The range of thermal neutrality for these animals is probably narrow, possibly because of the adaptation to nest conditions, where only modest fluctuations in the environmental temperature occur. This range of thermal neutrality probably changes parallel to the changes of “quasi” normal body temperatures. It is possible to establish neutral temperatures and to take them into account in experiments on respiratory metabolism.

Results obtained in experiments on respiratory or energy metabolism on the animals kept in temperatures other than neutral are difficult to interpret. Although such metabolism of infant rats is lower at 35°C than at 30°C (Antoschkina, '39; Fairfield, '48), the exact reason is not clear. It may be that the higher metabolism at 30°C is the result of extra heat production, since the temperature was below the low critical one; also, 35°C being close to the high lethal range may lead to a decrease in metabolism. The latter explanation is further supported by the fact that the "quasi" normal body temperature in rats up to 16 days after birth is below 35°C.

Adolf ('57), discussing the problem of measurements of respiratory metabolism in infant rats, explained that for the sake of uniform experimental condition the temperature of newborn animals should be artificially raised to that of adults, as was done, at least in part, by Antoschkina ('39) and Kibler and Brody ('42). The preceding discussion clearly shows the fallacy of this method of approach. Since we have shown that the "quasi" normal body temperatures in younger rats and the normal body temperatures in older rats are not the same at different stages of development, valid comparisons of the basal metabolism can be made only when the animals are allowed to maintain such temperatures; otherwise, one deals with a basal condition for the adults and a state of hyperthermia for the younger rats.

SUMMARY

Nelson strain rats of both sexes, from one to 20 days of age, were used for the determination of body (rectal) and skin temperatures in environments which ranged from 25 to 35°C. The results were described and the following conclusions were made:

1. Changes of environmental temperature within the range of 25 to 35°C did not produce in newborn rats any constriction or dilation of skin vessels. Such animals appeared to have no vasomotor mechanism of heat conservation and dissipation. This mechanism develops gradually from about the 4th day of postnatal life to, at least, the 20th day.

2. In spite of the lack of a mechanism for heat conservation, the body temperature of newborn rats was higher than that of the environment. Moreover, the difference between the temperature of the body and of the environment was much greater when the environmental temperature was 25°C than when it was 30°C.

3. Infant rats possess "quasi" normal body temperatures corresponding to the median body temperature of some primitive, so-called heterothermic mammals. These "quasi" normal body temperatures for rats, 1, 4, 8, 12, and 16 days old were 29, 30, 31, 33, and 35°C respectively.

Emphasis is placed on the need to use environmental conditions which permit the maintenance of the "quasi" normal or normal temperatures appropriate to each age group of experimental rats for valid comparison of metabolism. The point is made that previous studies of this type were actually conducted on infant rats in a state of hyperthermia.

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The Effect of Stretch on the Conduction Velocity of Single Nerve Fibers in *Aplysia*

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It seems well established (Gasser and Grundfest, '39; Hursh, '39; Tasaki, Ishii and Ito, '44; Berry, Grundfest and Hinsey, '44) that in vertebrate myelinated fibers, conduction velocity is a linear function of fiber diameter.

For non-myelinated fibers, Offner, Weinberg and Young ('40) and Hodgkin and Huxley ('52), proposed mathematical relationships, derived analytically, which were very similar and required conduction velocity in non-myelinated axons to be dependent on membrane resistance and capacitance, axoplasm resistance, and fiber diameter. Both these analytical approaches predicted that conduction velocity in non-myelinated fibers would be proportional to the square root of fiber diameter when all intrinsic membrane parameters were constant. Rushton ('51) felt this same relationship was implicit in the cable equation.

Pumphrey and Young ('38) working on *Loligo* and *Sepia* seemed to supply empirical confirmation of the velocity proportional to square root relation, but the very careful work of Hodes ('53) showed that in the third order giant of *Loligo*, conduction velocity is a linear function of fiber diameter. Gasser ('50, '55) using the method of reconstruction found the linear relation also held in non-myelinated fibers of mammalian peripheral nerves. Adey ('51) also found a linear relationship in the giant axons of the oligochete, *Megascolex*. However, Nicol and Whitteridge ('55) felt that some of their data on the median giant of the polychete, *Myxicola*, could be best described by the square root relation between velocity and fiber diameter. Their data, however, showed so much scatter that describing it by any such simple relationship is perhaps unjustified. Berkowitz ('55), using indirect methods, did find some empirical support for the square root relationship from the non-myelinated fibers of the lamprey, *Entosphenus*.

This work, as well as the work of Gasser and Erlanger ('27); Blair and Erlanger ('33); Zotterman ('37); and Eccles, Granit and Young ('32) is all in agreement that, not considering the shape of the function, conduction velocity is definitely a positive function of fiber diameter.

Bullock, Cohen and Faulstick ('50) measured the conduction velocity of the median giant of *Lumbricus* while holding the animal stretched at various lengths. With appropriate magnification and careful arrangement of background illumination, they were able to observe directly this fiber while recording from it. The unstretched fiber was coiled within the ventral cord. As the animal was stretched the fiber uncoiled and the velocity appeared to increase, without any changes in fiber diameter. However, when the fiber had been pulled out straight, further stretching reduced the fiber diameter up to one half its original value, but had no effect on conduction velocity. Martin ('54) found also in frog muscle that changes in length from 67% to 122% of reference length produced no changes in conduction velocity. Martin, using Offner, Weinberg and Young's equation, and Hodgkin ('54) proceeding directly from the cable equation, independently proposed explanations of these surprising findings based on the postulate that the stretch would be taken up by the smoothing of submicroscopic folds. Both these explanations required that the area of the axon membrane would not change during stretching of the axon.

The present experiments extend the findings of Bullock, Cohen and Faulstick to another group of animals, and demonstrate the phenomenon on a preparation which, at least in certain respects, seems more favorable for quantitative study.

MATERIALS AND METHODS

All this work was done on 20 specimens of the tectobranch, *Aplysia californica*. In each case, either the left or the right pleuro-visceral connective and its ganglia was removed from the animal, and its length under zero tension was recorded. The connective was then clamped just posterior to the pleural and anterior to the visceral ganglia. The clamps were arranged so that the distance between them could be varied by small increments. Conduction velocity was recorded in terms of delay between two fixed pairs of silver wire macro-recording electrodes, separated by about 1.5–2.0 cm. Signals were led off to an AC coupled amplifier and dual beam oscilloscope. Delays were measured between the peaks of the two diphasic spikes from photographs of the oscilloscope screen. Figure 1 is typical of the records from which velocity measurements were taken. Square wave stimuli of 0.2 msec. duration were delivered from a pair of silver wire stimulating electrodes whose poles were separated by about 2 mm. The stimulating electrodes were never placed within 0.3 cm of the recording electrodes closest to them. The length of these connectives at zero tension ranged from 2.0 to 3.5 cm. Wet weight ranged from 125 to 275 gm.

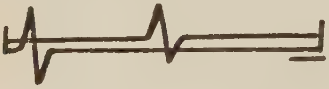


Fig. 1 Record of R3 recorded at 0.3 and 2.3 cm from the stimulating electrodes. The stimulus artifact is at the left. The length of the connective is 3.5 cm (71% stretch). Calibration, 200 μv, 5 msec.

RESULTS

The lowest threshold response of the right pleuro-visceral connective to electrical stimulation on that connective is a relatively fast conducting, low amplitude, all or none spike (RI). When the connective was clamped at a length just sufficient to prevent visible slack, but with no stretch on it (zero tension), RI measured 200 μv in amplitude. In this and in all other fibers studied, the spike amplitude increased by several fold as the connective was stretched out. This is probably due to a decrease in the amount of shunting tissue between the active membrane and the recording electrodes. By raising the stimulus intensity, a second, slower conducting, larger (400 μv at zero tension), all or none spike (R2) is observed. The lowest threshold response of the left pleuro-visceral connective to electrical stimulation on that connective is another all or none spike (R3).

The conduction velocity was measured for R1, R2, and R3 as a function of length of the connective. The results of all measurements on any one of the three fibers are plotted on the same graph. For each specimen velocities were recorded for each length of the connective. As the length of any connective increased from the unstretched length, the first few velocity measurements would each be greater than the preceding, these values increasing with increasing length to a maximum value. Velocity would not differ from this value appreciably at any length of the connective greater than that at which this maximum value was first reached, regardless of how far the connective was stretched. For each specimen the mean maximum value velocity (the average of velocity measurements obtained at all lengths greater than that at which maximum value velocity is first observed) was calculated and all velocities from that specimen plotted as per cent difference from this value. Lengths are plotted as per cent increase in length over resting length (i.e., per cent stretch). Resting length is the recorded length at zero tension. The results are plotted in figures 2(R1), 3(R2), 4(R3); the pooled measurements of 12, 7 and 6 specimens, respectively.

These data may be represented by the intersection of two straight lines, one of positive, and the other of zero slope. In R1 and R2, velocity is independent of stretch from 40% to 325% stretch, in R3 from 70% to 425%.

When the conduction velocity of a fiber was measured at constant per cent stretch, as a function of time (fig. 5), the variability in conduction velocity over a period of time which was twice as long as that of a typical stretch experiment, was as great as that in figures 2, 3 and 4, indicating that any deviation of points from a straight line in figures 2, 3 and 4 is within the experimental limits of reproducibility and not a systematic function of stretch.

In each case, the fiber conducted until the connective broke. These curves were not reversible, as releasing tension at any high per cent stretch would cause a decrease in the length of only a few per cent.

The mean absolute velocities, calculated by averaging the mean maximum value velocities (flat part of the stretch-velocity curve) for each specimen for which there is data on that fiber, are recorded in table 1. Temperature varied between 17.5 and 20°C.

CONCLUSIONS

Tauc ('57), using *Aplysia depilans*, stimulated the largest cell in the right visceral ganglion with intracellular electrodes and recorded extracellularly on the right pleuro-visceral connective. He showed that the largest spike from the right pleuro-visceral connective was produced by a process from the largest cell in the right visceral ganglion, and hence, presumably from the largest fiber in the connective.

The discrepancy which R1 and R2 exhibit with respect to the usual conduction velocity relationship (a positive function of fiber diameter) may be ascribed to differences in intrinsic membrane and axoplasm constants. This points to the possibility of error inherent in an approach which tries to compare velocities in different fibers by assuming that intrinsic characteristics are constant.

If these fibers behave like the median giant of the earthworm, then the rising portion of the stretch-velocity curves is due to uncoiling of the fibers, reducing the actual conducting distance between the fixed recording electrodes, and producing an apparent increase in velocity. In fact, the nervous portions of the connectives at zero stretch are tubes twisted into helices lying within a cylinder of connective tissue which, when viewed histologically, forms a well defined capsule surrounding the nervous tissue proper.

The adaptive value of this portion of the curve to an animal which can change its body length by a factor of two is obvious. The author has never observed these connectives stretched more than 70% when in the animal. If the uncoiling hypothesis is correct, then the total conducting time between the pleural and visceral ganglia should not change over the rising portion of the curve, and in nature should remain constant.

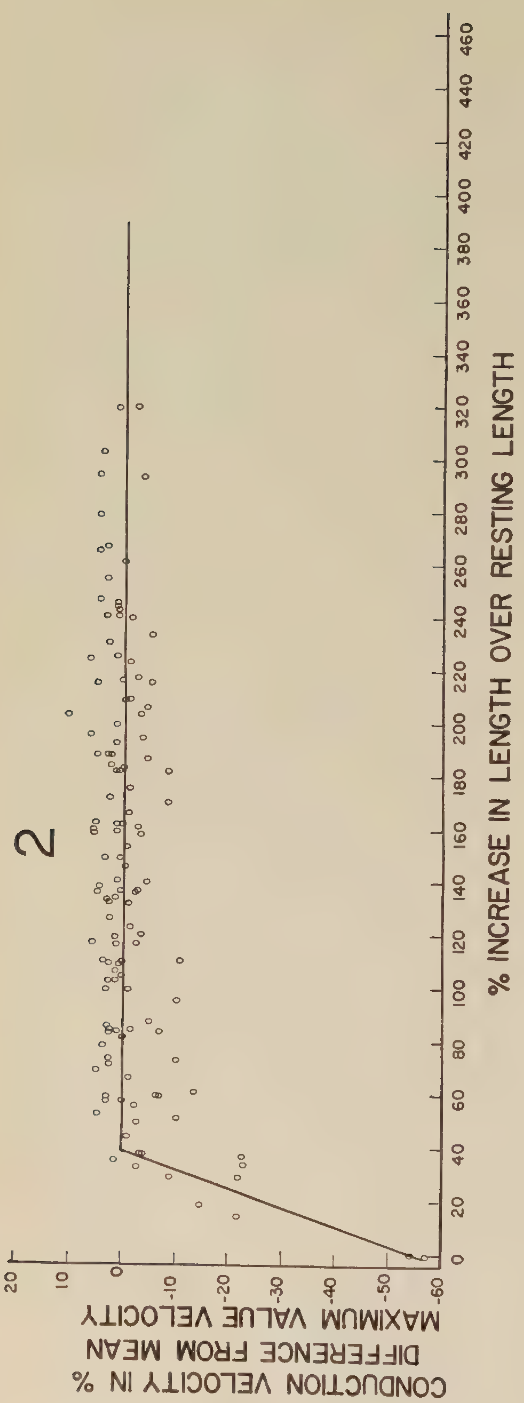
If we regard the axon as a cylinder of uniform diameter, then, since the membrane is very thin compared to the axon diameter, the axoplasm volume (V) will be given by

$$V = \pi r^2 L$$

where r is the axon radius and L, its length. For R1, if the uncoiling hypothesis is correct, L is changing only when the fiber is being stretched from 40% to 325%. At 40% stretch, let $L = L_1$. Then at 325% stretch, $L = L_2 = 3.03\ L_1$. If there were no diameter changes over this range of stretch, then the axoplasm volume would have to increase 3.03 times. Similarly for R2, the volume would have to increase 3.03 and for R3 3.09 times. As an increase in axoplasm volume is unlikely the diameter must have decreased as the fiber was stretched from L_1 to L_2. It is likely, then, that these fibers behave like the median giant of *Lumbricus*, and that over the flat portion of the stretch-velocity curve diameter is decreasing, and conduc-

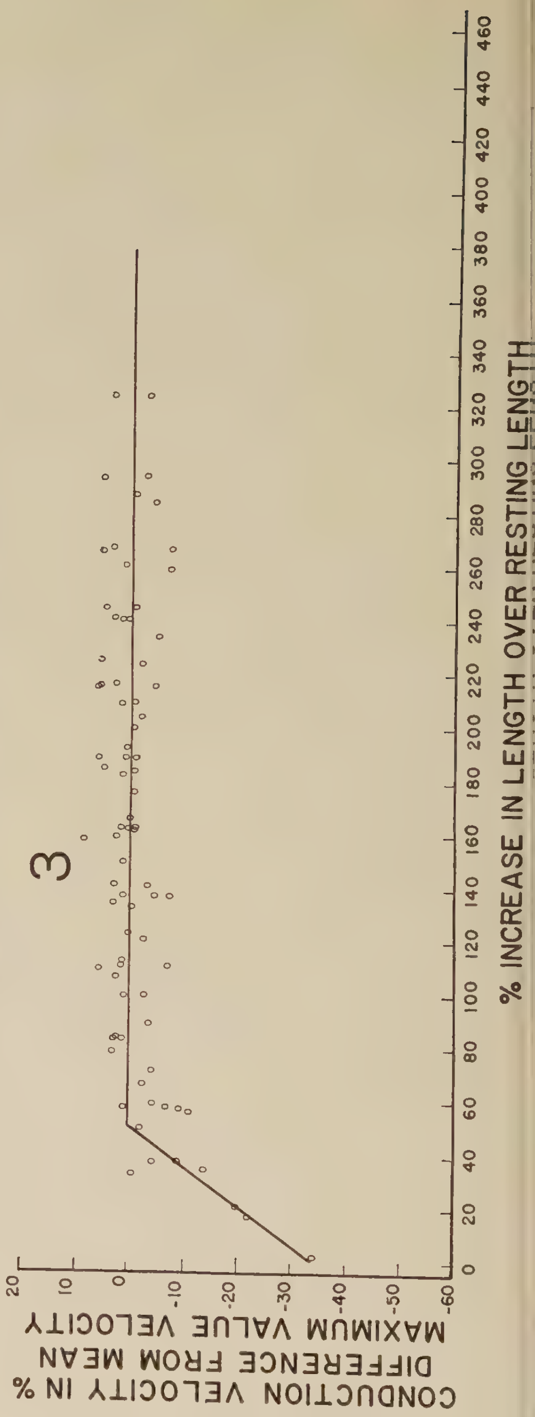
TABLE 1

Fiber	Mean maximum value velocities averaged for all specimens used	Range in mean maximum value velocity among different specimens	Number of specimens data collected from
	cm/sec.	*cm/sec.*	
R1	91	75–108	12
R2	50	43–55	7
R3	114	98–136	6



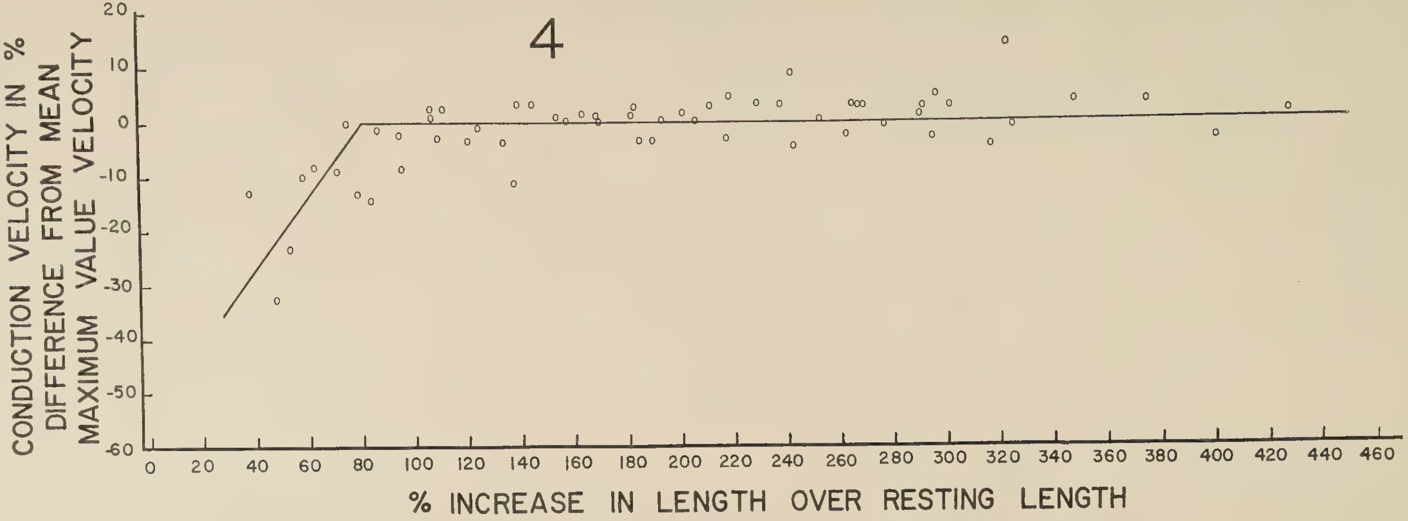
2

CONDUCTION VELOCITY IN % DIFFERENCE FROM MEAN MAXIMUM VALUE VELOCITY
20
10
0
-10
-20
-30
-40
-50
-60
% INCREASE IN LENGTH OVER RESTING LENGTH
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460



3

CONDUCTION VELOCITY IN % DIFFERENCE FROM MEAN MAXIMUM VALUE VELOCITY
20
10
0
-10
-20
-30
-40
-50
-60
% INCREASE IN LENGTH OVER RESTING LENGTH
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460



4

Fig. 2 Plot of conduction velocity in per cent difference from the solid line of zero slope (see text) vs. length of the connective in per cent increase in length over resting length for the R1 response of the right pleural-visceral connective in *Aplysia californica.* Each circle represents a single velocity measurement. Data collected from 12 specimens. Eighty-five per cent of the circles between 40 and 320% stretch fall within ± 5% of the solid line of zero slope.

Fig. 3 Plot of conduction velocity in per cent difference from the solid line of zero slope (see text) vs. length of the connective in per cent increase in length over resting length for the R2 response of the right pleural-visceral connective in *Aplysia californica.* Each circle represents a single velocity measurement. Data collected from 7 specimens. Eighty-six per cent of the circles between 40 and 320% stretch fall within ± 5% of the solid line of zero slope.

Fig. 4 Plot of conduction velocity in per cent difference from the solid line of zero slope (see text) vs. length of the connective in per cent increase in length over resting length for the R3 response of the left pleural-visceral connective in *Aplysia californica.* Each circle represents a single velocity measurement. Data collected from 6 specimens. Ninety-two per cent of the circles between 80 and 420% stretch fall within ± 5% of the solid line of zero slope.

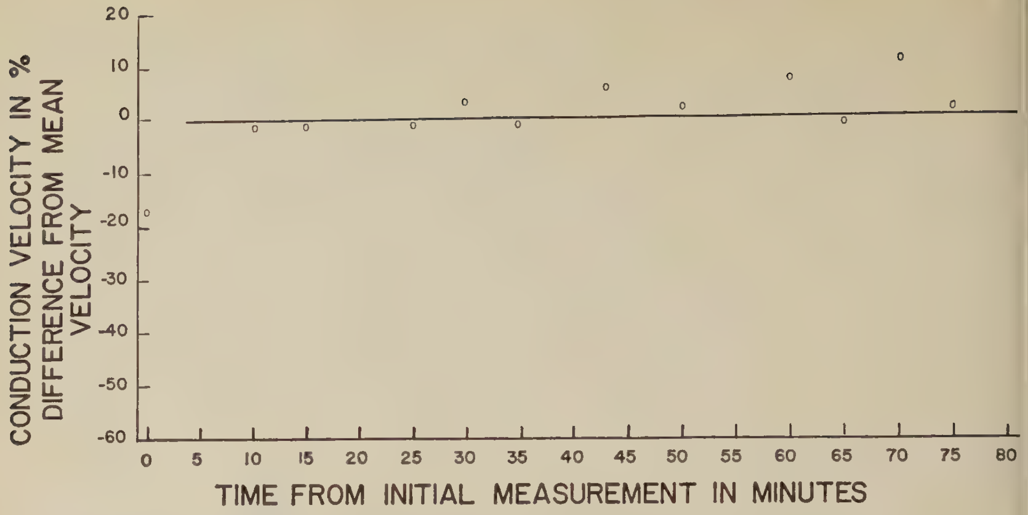


Fig. 5 Plot of conduction velocity in per cent difference from the solid line vs. time for the R1 response of the right pleural-visceral connective in *Aplysia californica.* Each circle represents one velocity measurement. These data were taken from a single preparation held at 70% stretch.

tion velocity is independent of fiber diameter.

In Martin's unfolding model

$$r \alpha L^{-0.5}$$

then, if $r = r_1$ where $L = L_1$, and $r = r_2$ where $L = L_2$, and since L_2 is approximately equal to $3L_1$

$$r_2 = 3^{-0.5} r_1.$$

For a cylinder of uniform diameter

$$A = 2\pi r L$$

Where A is the surface area. In Martin's model surface area is constant over that range of length where velocity is constant. If we assume at L2, i. e., the point at which the fiber breaks, all the membrane folds have been pulled out straight and the axon is a smooth bored tube, then

$$A = 2\pi r_2 L_2 \text{ or}$$
$$A = 2\pi 3^{-0.5} r_1 L_1$$

The apparent area at L_1 (A_1) is given by

$$A_1 = 2\pi r_1 L_1$$

Then, at L_1 A_1 is only 58% of A, i.e., 42% of the membrane would be occupied in foldings.

Martin noted that capacitance per unit apparent area and conductance per unit apparent area would both be functions of fiber length over that range of length in which the fiber membrane was unfolding. In Martin's view conduction velocity would appear to be independent of fiber length and hence diameter because the length changes were inducing changes in capacitance and conductance which would exactly compensate for one another. Certainly his model and the equation upon which it is based require measurements of membrane capacitance and conductance as functions of stretch for full confirmation. Until this is done the observations of Bullock, Cohen and Faulstick and those presented here will not be capable of reconciliation with that large body of data which establish velocity as a positive function of diameter.

SUMMARY

1. Measurements were made on the effects of stretch on two fibers in the right and one fiber in the left pleuro-visceral connective.

2. It was possible to change the length of these connectives 300% to 350% without a change in conduction velocity.

3. It is expected that over this range conduction velocity is not a function of fiber diameter in these three fibers.

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ADDENDUM

Since this paper went to press the work of Turner ('51) on the pedal nerves of the slug *Ariolimax* was called to my attention. Turner was able to stretch these nerves to twice their resting length without a change in conduction velocity of the fastest response. The amount of folding that Turner observed histologically in the sheath of the unstretched largest fibers is not sufficient to account for the increased length on the basis of Martin's model, supplying direct evidence on at least this one preparation that the constant surface area model is inadequate to explain the observed velocity-diameter relationship.

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Calcium Influx in Frog Rectus Abdominus Muscle at Rest and During Potassium Contracture

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In a survey of recent literature the proposal was revived that a transitory rise in the intracellular calcium ion concentration may underlie the initiation of contraction and contracture in different kinds of muscles under a wide variety of conditions (Shanes, '58). By certain modifications in familiar radioisotope techniques, Bianchi and Shanes ('59) were subsequently able to detect and measure relatively small increases in calcium influx that occur in frog sartorius muscle with activity. Although small, the increase in influx estimated per impulse (and twitch) over a square centimeter of membrane was 30-fold greater than previously reported for nerve fibers—the giant axons of the squid (Hodgkin and Keynes, '57)—whereas the influx in the unstimulated muscle and nerve fibers was about the same. Moreover, an increase in the strength of the twitch of the individual fibers, as may be brought about by replacing the chloride of the medium with nitrate (Kahn and Sandow, '55), was accompanied by the same percentage increase in calcium influx per twitch. Recently, the linearity between contractility and the calcium influx during each contraction has been strikingly demonstrated in our laboratory by Winegrad ('60) in studies of the treppe phenomenon and of the action of calcium in guinea pig atria. These recent findings therefore support the initial suggestion as to the role of calcium and indicate in addition that the strength of contraction is a direct function of the calcium entering the cell during the impulse. A similar direct relationship between the monovalent ions and contraction has not been found (e.g., Reiter, '56).

In addition to the *quantitative* correlation between calcium influx and the strength of contraction induced by electrical stimulation, Bianchi and Shanes ('59) observed a *temporal* correlation between influx and potassium contracture. Thus, the sartorius fibers, which are solely of the "twitch" type (Gray, '58; Krüger, '52; Krüger et al., '33), produce only a transitory rise in tension when the potassium level of the medium is raised even though the fibers remain depolarized as long as the excess potassium is present (Hodgkin and Horowicz, '60; Krüger, '52). In keeping with this, a large augmented influx of calcium occurred only at the time the potassium concentration was first raised; none could be detected shortly thereafter.

A further test of the validity of the temporal correlation between calcium influx and potassium contracture is provided by "slow" fibers, which differ from twitch fibers in producing a sustained contracture (Krüger, '52; Krüger et al., '33; Kuffler and Williams, '53). In these one would expect the influx to remain elevated. This will be shown to be the case. In addition, the effects of a cardiac glycoside and calcium will be described. A preliminary report of these findings was presented at the Fall, 1960 meeting of the American Society for Pharmacology and Experimental Therapeutics (Shanes, '60).

METHODS

The *M. rectus abdominis* is particularly rich in slow fibers; however, they may still represent fewer than 30% of the total (Krüger, '52; Krüger et al., '33; Kuffler and Williams, '53). This tissue, taken chiefly from gravid *Rana pipiens* females from April through July, was used throughout. To minimize the participation of twitch fibers in the phenomena to be described, 10 mg% cocaine hydrochloride was present in all solutions and potassium concentrations did not exceed 32 mM (Bianchi and Shanes, '59; Hodgkin and Horowicz, '60a; Krüger et al., '33).

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Both halves were dissected out separately and as carefully as possible. The need to remove the sternum for efflux studies because of its large uptake of radiocalcium, and the poorly defined boundary between the lateral edges of the halves and the adjacent abdominal muscles introduced uncertainty as to the amount of tissue damaged in these regions as a result of dissection. As will be pointed out later, there is reason to believe this was small. For influx determinations, which are the major concern of this report, the excessive uptake of calcium by any deteriorating fibers (Gilbert and Fenn, '57) presented no problem since the questionable regions were removed prior to application of analytical procedures.

Paired halves of the rectus abdominis muscle were used separately as control and experimental preparations. The distal end of each muscle was secured by a very fine piano wire clip[1] to the end of a fine glass tube that served to immobilize and to oxygenate the solution bathing the muscle. The proximal end was attached to a fine tantalum wire with the aid of a second clip. The tantalum wire was connected to an isotonic ink writing lever which loaded the muscle with a total of 2 gm. Two and a half milliliters of solution in a small test tube could quickly be placed in contact with the tissue or replaced in 5 seconds. In this manner each half of rectus muscle was exposed to radiocalcium solution, frequently preceded by special preparatory solutions, and then washed at regular intervals thereafter in 2.5 ml non-radioactive solutions replaced completely at 3, 8, 18, 28, 48, 68, 88 and 118 minutes to empty the interstitial space of radiocalcium. Each washout solution was dried and counted to obtain the washout (desaturation) curve.

As in the previous studies (Bianchi and Shanes, '59), the uptake of Ca^{45} served for estimates of calcium influx, the sensitivity of the method being increased by keeping the period of exposure to radioisotope short and by clearing the extracellular space of radioisotope prior to preparation for counting. The potassium level of the medium was increased by adding KCl in excess to the Ringer's solution. Other additions to the Ringer's solution were by replacement of the NaCl.

After washout the tissues were blotted, weighed, dried, reweighed (for water determinations), and then ashed at 500°C for 16 hours; the residue was treated for 3 to 4 hours with acidified Ringer's solution and this then deposited and dried on planchets for counting (Bianchi and Shanes, '59).

The normal and radioisotope-containing Ringer's solutions employed were the same as in earlier studies (Bianchi and Shanes, '59), i.e., they contained 1 mM Ca^{++}, 1.6 mM K^+ as well as NaCl and a slight amount of phosphate buffer; Ca^{45}, when present, was at a level of 1 microcurie/ml.

Most experiments were carried out at room temperature (23–24°C); they were begun within an hour of removal of the muscles from the animals.

RESULTS

Kinetics of Ca^{45} release. Preliminary measurements of Ca^{45} loss were carried out on muscles previously soaked for two hours in radioactive Ringer's solution chiefly to set the experimental conditions and to obtain approximate correction factors for evaluating influx. Figure 1 gives a typical curve obtained with paired muscles. It shows the usual two distinct components—fast and slow; as usual, the fast component will be regarded as extracellular in origin, the slow component as derived from within the fibers (Bianchi and Shanes, '59; Gilbert and Fenn, '57; Shanes and Bianchi, '59). In 4 muscles the total Ca^{45} space averaged 1.08 ml/gm, the zero time intercept 17%, and the half-time 3–5 minutes for the fast component and 91 minutes for the slow component. The water content of 6 muscles averaged 80.7%. The total space, the half-time of the fast component, and the water content are about those of sartorius muscle; the zero time intercept and the half-time of the slow component are appreciably smaller (Shanes and Bianchi, '59). The

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[1] Orginally designed by S. Winegrad for use on guinea pig atrial appendages for attachments with negligible cellular damage.

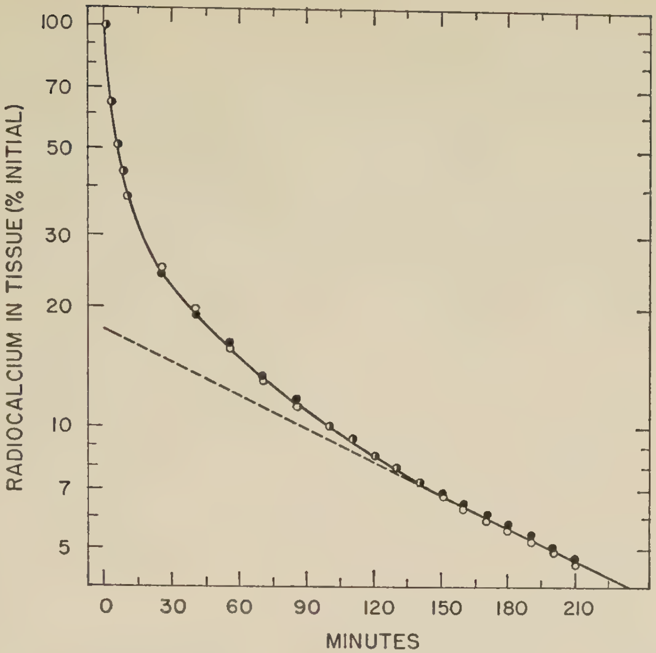

Fig. 1 The time course of decline of Ca^{45} in paired halves of the rectus abdominis muscle of the frog washed continually in radiocalcium-free Ringer's solution. Both muscles were exposed to radiocalcium-containing Ringer's solution for two hours prior to washout.

slow exponential decline becomes evident in about 120 minutes—apparently the time of completion of emptying of the extracellular space—hence two hours was routinely employed to wash out interstitial radioactivity prior to analyzing for the activity in the fibers for influx determinations.

Since the calcium concentration of the medium was 1 μmol/ml, the space and intercept indicate an exchange with and/or an uptake of 0.18 μmol/gm calcium intracellularly and 0.90 μmol/gm extracellularly. The latter is appreciably larger than in sartorius muscle (Shanes and Bianchi, '59) and could be the consequence of (a) a larger amount of interstitial tissue water, (b) greater binding of calcium by connective tissue and/or the superficial sites on the muscle fibers, and (c) an initial large uptake of Ca^{45} by damaged fibers.

If the half-time for Ca^{45} uptake by the fibers equals that for the washout—91 minutes—the intracellular calcium exchange is incomplete in the two hours employed. Assuming a simple exponential uptake, we can easily show that in infinite time the exchange and uptake would have been 0.30 μmol/gm instead of 0.18. This is appreciably less than the corrected value estimated for sartorius muscle (Shanes and Bianchi, '59).

For a uniform population of fibers with a half-time of Ca^{45} loss of 91 minutes and with the above content of exchangeable calcium the outflux is 2.3×10^{-3} μmol/gm min. Slow fibers in the ileofibularis muscle average about 50 μ in diameter (Gray, '58); twitch fibers average about 100 μ in the sartorius muscle (Carey and Conway, '54). An estimate of the surface area of the rectus muscle must probably take into account different average areas as well as the presence of an interstitial space. If the latter is considered to be 20%, we obtain a surface area in the neighborhood of 400 cm^2/gm. Using this figure, we estimate the calcium outflux to be 0.096 μμmol/cm^2 sec, which approximates the value of 0.094 obtained for outflux and influx in sartorius muscle (Bianchi and Shanes, '59; Shanes and Bianchi, '59). Such agreement, if more than fortuitous, may indicate that damaged fibers were not very numerous in the rectus prep-

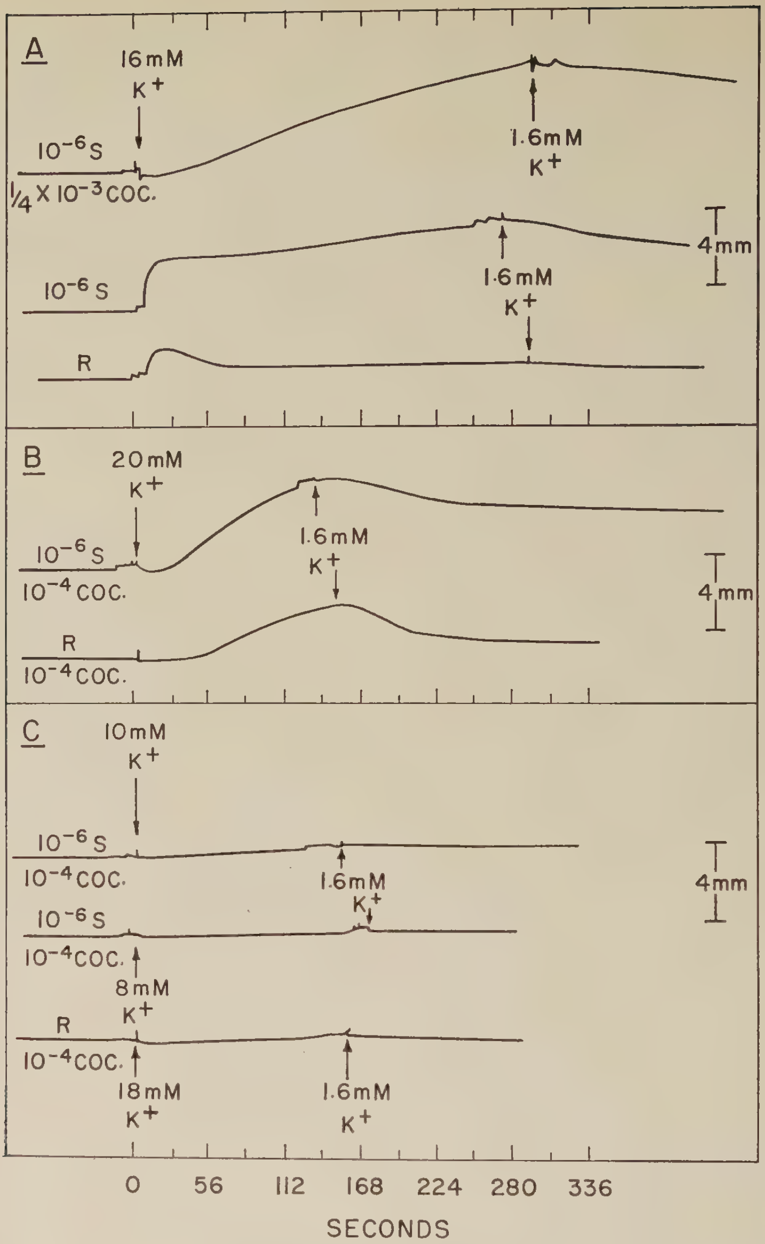

Fig. 2 A, comparison of the shortening of the same half of rectus muscle in response to 16 mM KCl (a) added to Ringer's solution (R), (b) after exposure for 15 minutes to Ringer's solution containing 10^{-6} (w/v) K-strophanthin (S), and (c) after 50 minutes exposure to K-strophanthin and 13 minutes treatment with cocaine (COC.). B, comparison of the latent period and rate of shortening of a rectus muscle in response to 20 mM KCl before (R) and after 15 minutes' treatment with 10^{-6} K-strophanthin (S). C, comparison of the threshold level for potassium contracture before (R) and after addition of K-strophanthin (S) to the Ringer's solution. At 8 mM, KCl causes no response in strophanthin in the allotted time, but at 10 mM it causes about the same weak response as 18 mM in the absence of strophanthin.

arations and hence did not appreciably affect the present results.

Features of isotonic contraction. Since in all flux experiments potassium contractures were recorded, it was possible to note several of their features. It was soon apparent, before cocaine was routinely employed, and as emphasized by Lorković ('59b), that there is an initial rapid response followed by a slower shortening; the first phase often begins to decline before the second is apparent (fig. 2). As noted by Krüger et al., ('33), the first phase is like the response of twitch fibers in that it can be suppressed by cocaine (fig. 2A). Repetitive firing of the twitch fibers in the rectus abdominis may be partly responsible for the first stage. In figure 2A it is evident that strophanthin augments the first as well as the second stage of contracture in potassium. All future studies were carried out with 10 mg% cocaine present in the medium.

In cocaine containing media there was typically a phase of rapid shortening in elevated potassium which blended continuously into a long phase of gradual shortening. The figures given do not show the slow phase because normal potassium concentration (1.6 mM) was restored too early. It cannot be ruled out that the fast phase represents at least in part contraction by twitch fibers, relaxation being prevented by the slow fibers in parallel that take up and sustain the shortened state. Studies with single fibers would clarify the situation.

At the potassium concentrations used in these studies, a distinct latent period was evident before contracture began to develop. In general, an increase in contracture, such as induced by higher potassium concentrations or by a cardiac glycoside and by thiocyanate, was accompanied by a shortened latent period; a weakened contracture, such as caused by an elevation in extracellular calcium, was associated with a lengthened latent period. In figure 2B it is apparent that a latent period of 30 seconds in 20 mM K is reduced to about half by prior exposure for 15 minutes to 10^{-6} (w/v) K-strophanthin. This clearly suggested a lowering of the threshold concentration, that is, the minimum concentration of potassium required to produce a contracture. This was easily confirmed, as shown in figure 2C. In these instances, the response obtained in 18 mM K^+ was duplicated by 10 mM K^+ after 15 minutes treatment with 10^{-6} K-strophanthin. Figure 3A shows that 11 mM SCN^- acts similarly to the glycoside; thus, 9 mM K^+ with SCN^- present causes the same contracture as 18 mM K^+ alone. Lowering of the K^+ threshold of frog sartorius fibers by SCN^- and NO_3^- was demonstrated by Hodgkin and Horowicz ('60), and of turtle retractor penis by I^- was shown by Curtis and Csapo ('58).

In table 1 it is apparent that the extent of rapid shortening varied inversely with extracellular calcium concentration. An inhibitory effect by calcium on potassium contracture has been noted before (Fleckenstein et al., '50; Kraatz, '60; Lorkovič, '59b). In some cases merely the final amplitude and not the rate of shortening was markedly altered. Figure 3B shows an experiment in which quadrupling the calcium concentration *after* K had been applied slowed the rate of shortening proportionately, while a return to the original calcium concentration led to a resumption of a higher rate of shortening; the last effect is small by virtue of its occurrence during the phase of slow shortening. The action on the rate, as well as a lengthening of the latent period (because of an elevated contracture threshold, as shown by Lorković, '59b), is reflected by the 50% increase in the time from potassium application to attainment of half the maximum quick shortening (table 1).

Calcium influx. Since exposure of the rectus muscles to Ca^{45} Ringer's solution for 5 or 9 minutes gave no significantly different estimate of the passive influx,

TABLE 1

Comparison of the amplitude, h, and half-time, t,[1] of the rapid phase of contracture in 32 mM K^+ at two concentrations of extracellular calcium

$[Ca]_o$	h[2]	t[2]
mM	*mm*	*sec.*
1	10.9 ± 1.7	3.5 ± 0.57
3	3.4 ± 0.64	5.6 ± 0.57

[1] Measured from application of K^+, therefore includes latent period.

[2] Six preparations contributed to each mean and its standard error.

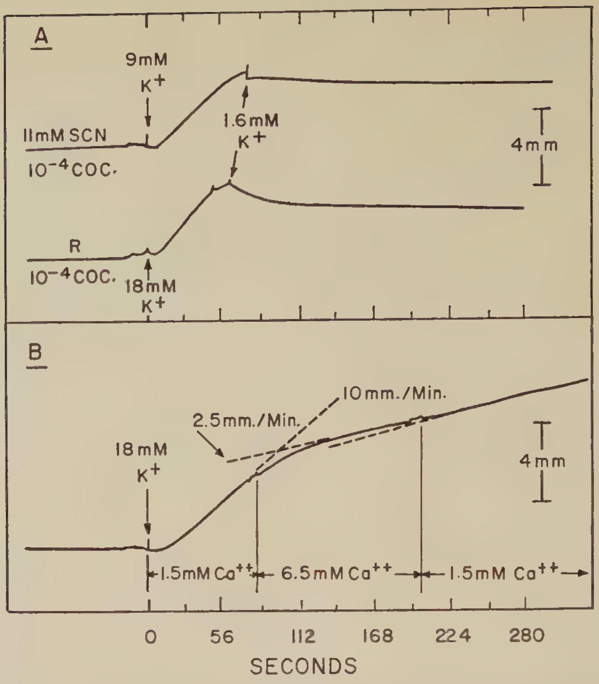

Fig. 3 A, comparison of the response of a rectus muscle to 18 mM KCl in Ringer's solution (R) and to 9 mM KCl 17 minutes after the addition of 11 mM SCN⁻ to the medium. B, changes in the rate of potassium contracture upon elevation and subsequent lowering of extracellular calcium concentration.

the shorter interval was regularly employed. The effect of K⁺ on Ca^{45} uptake was observed by adding K⁺ for one minute before, simultaneously with, or 4 minutes after addition of Ca^{45}, the exposure to K⁺ being otherwise together with Ca^{45}, usually for 4 or 5 minutes. When the effect of calcium concentration was under study, the change in concentration occurred 4 minutes before the application of K⁺ and Ca^{45}. As pointed out in Methods, in every instance washout in non-radioactive solutions for 120 minutes preceded estimation of the retained Ca^{45}. While this had the salutary effect of eliminating the obscuring effect of extracellular Ca^{45}, it also reduced the absolute estimate of the Ca^{45} uptake prior to washout because of loss from the fibers. This and other factors tending to give an underestimate of influx will be considered later. The uncorrected data, given in table 2, nevertheless allow important comparisons to be made. Thus, at an extracellular calcium concentration of 1 mM, a ten-fold increase in extracellular K⁺—to about the threshold for contracture—trebles the uptake of calcium by the fibers. This is the case whether Ca^{45} is applied to the tissue prior to K⁺ or with it. Trebling the extracellular calcium level trebels the influx of calcium at normal (1.6 mM) K⁺ concentration, as noted in the sartorius muscle (Bianchi and Shanes, '59), but has no effect on the influx at the next higher K⁺ concentration; the effect of a 10-fold increase in $[K]_o$ to 16 mM on calcium influx is therefore negligible at the higher calcium concentration. However, at both calcium levels, an additional doubling of $[K]_o$, which induces a clearcut contracture, substantially increases calcium influx.

Interesting differences in the estimates of influx are apparent according to whether Ca^{45} is allowed to penetrate the interstitial spaces before K⁺ is applied (in the continued present of Ca^{45}) or whether it is applied only concurrently with K⁺ or after K⁺. Prior treatment with Ca^{45} leads to a substantially greater estimate of calcium uptake than the other two situations; both of the latter give essentially the same smaller

TABLE 2

Calcium influx (uncorrected) of frog rectus abdominis muscle[1]

Values given are in (μmol/gm min.) × 10^{-4} and are those obtained at different external potassium levels, $[K]_o$, with extracellular calcium concentration, $[Ca]_o$, at normal (1 mM) and elevated (3 mM) values; the influence of applying Ca^{45} before, with, and after the increase in $[K]_o$ is also shown (see text).

$[K]_o$ in mM:	1.6[2]	16[3]		32		
Ca^{45} relative to K:		Before	With	Before	With	After
Influx ($[Ca]_o$ = 1 mM)	3.15 ± 0.35(13)	9.56 ± 0.84(9)	7.9 ± 2.6(2)	33.9 ± 6.4(8)	12.4 ± 1.3(6)	14.6 ± 1.7(4)
($[Ca]_o$ = 3 mM)	9.4 ± 2.6(5)		8.3 ± 3.6(2)	29.5 ± 2.1(4)	19.0 ± 2.3(3)	

[1] Numbers in parentheses give the size of the sampling.

[2] Concentration in Ringer's solution.

[3] Approximately threshold concentration.

influx figure. It will be recalled that in twitch fibers, where K^+ application causes a transitory contracture, only prior Ca^{45} treatment reveals a striking increase in calcium influx; Ca^{45} applied with K^+ gave no indication of an increase in influx (Bianchi and Shanes, '59). From this it was concluded that twitch fibers exhibit increased influx only during the initial period of depolarization, as might be expected if calcium entry were a determining factor in the temporal features of the K^+-contracture. Since, as already pointed out, it is likely that twitch fibers are present in the rectus abdominis muscle, it is possible that the larger uptake observed with prior Ca^{45} treatment reflects the response of the twitch fibers. Of special significance in the present results is the fact that the influx *remains* elevated with continued exposure to K^+, being 4-fold greater than in 1.6 mM K^+ at the normal (1 mM) calcium level and about two-fold greater when $[Ca]_o$ is 3 mM. This is what would be expected if sustained contracture in slow fibers is also dependent on a sustained intracellular level of ionized calcium—since this would follow from a stationary state between calcium entry and subsequent binding—and if the reduced contracture in elevated $[Ca]_o$ reflects the smaller increment in calcium *relative* to the influx in 1.6 mM K^+.

It should be emphasized that the comparison of the calcium influx in high (32 mM) K^+, obtained when Ca^{45} is applied with (or after) excess K^+, is made with that in 1.6 mM K^+ because such was the comparison made earlier in twitch fibers which revealed no difference in the influxes at high and low K^+ concentrations (Bianchi and Shanes, '59). Smaller increments in K^+—insufficient to induce contracture—caused a substantial sustained increase in calcium influx in the twitch fibers (Bianchi and Shanes, '59), with which the sustained increase in 16 mM K^+ at normal calcium levels shown in table 1 may be compared. Inasmuch as the twitch fibers in the rectus abdominis probably contributed substantially to this elevated influx, comparison of the *sustained* influx with the 16 mM influx is not a valid index as to whether the sustained values, more clearly due to slow fibers, are

TABLE 3

Increase of calcium influx of rectus abdominis muscle in 16 mM $[K]_o$ by 4 minutes prior treatment with K-*strophanthin* (10^{-6} *w/v*)

Control (C)	Stroph (S)	S/C
µmol/gm min. $\times 10^{-4}$	*µmol/gm min.* $\times 10^{-4}$	
7.8	8.6	1.1
5.7	12.0	2.1
15.0	23.0	1.5
8.4	10.0	1.2
12.0	18.0	1.5
Average		1.5

elevated above that of the *same* fibers in 16 mM K^+.

We have already noted the enhancement of K^+ contracture by K-strophanthin and thiocyanate, an effect seen to be a consequence of a lowering of the threshold potassium concentration. It was therefore of interest to compare calcium influx upon addition of potassium at threshold concentration (16 mM) in the presence and absence of K-strophanthin. In table 3 it is apparent that in the 5 paired preparations tested the influx averaged 50% greater in strophanthin. Washout experiments on these preparations gave no evidence that the rate of calcium outflux was altered by the glycoside.

The elevation of calcium influx in K-strophanthin is of interest inasmuch as it provides a basis for the lowering of the threshold potassium concentration for contracture. Thus, if a critical rate of calcium entry underlies the level of the threshold, as suggested by previous correlations, it follows that when an agent such as strophanthin raises the rate of entry for a given potassium concentration, a lower extracellular potassium level will now be required to achieve the same response. Bianchi ('61) has provided data that show an elevation of K^+ induced calcium influx when chloride is replaced by nitrate, in keeping with the augmentation of K^+-contracture under these conditions (Hodgkin and Horowicz, '60); a similar correlation between enhancement of influx and of the twitch by nitrate was demonstrated previously (Bianchi and Shanes, '59).

DISCUSSION

The present studies extend still further the correlation between calcium influx and contraction. The temporal correlation in twitch fibers between the *transitory* increase in calcium influx with the *transitory* K^+-contracture is reinforced as a cause-effect relationship with the current finding that in slow fibers a *sustained* increase in calcium influx accompanies a *sustained* contracture. Moreover, the quantitative correlation established in sartorius and atrial fibers is strengthened further by the finding that increased sensitivity to potassium in strophanthin is associated with an increased influx of calcium.

That elevation of $[Ca]_o$ reduces the contractile response of the rectus muscle poses an important problem for the hypothesis postulating that the level of ionized calcium within the fibers is a controlling factor. In twitch fibers the passive entry of calcium increased in proportion to $[Ca]_o$, whereas an increase in influx with activity could not be detected, in keeping with the negligible change in twitch height (Bianchi and Shanes, '59). In the rectus muscle, on the other hand, where the contracture is reduced by elevation of $[Ca]_o$, the response with respect to calcium uptake seems little different from the twitch fibers. However, certain observations raise the possibility that this similarity in the calcium uptake characteristics is superficial and that important differences may be present with respect to calcium distribution and hence its effect on shortening.

Thus, two studies have shown that complete absence of extracellular calcium for several hours can obliterate the initial (Schaechtelin and Lüttgau, '60) and especially the sustained contracture (Denton, '48) and that preparations previously depleted of calcium give a progressive increase in the contracture with increasing [Ca] up to a certain optimum (e.g., 4–8 mM Ca^{++}). Denton ('48) states that the prolonged contracture of these muscles is independent of calcium at higher concentrations. Hence it appears that prior emptying of calcium from the fibers gives results in better keeping with the calcium hypothesis than our own or those of Kraatz ('60), Lorković ('59b), and Fleckenstein et al. ('50), all of whom did not deplete the fibers of calcium before their experiments with elevated $[Ca]_o$.

A possible basis for the effect of prior lcium depletion is seen in the finding by eachey and Huxley ('60) that in slow ers the spread of contractile activity iginates from localized sites of depolaration in both A and I bands and spreads th radially and longitudinally in conast to the situation in amphibian twitch ers, where activation spreads only radily and occurs only in quite independent bands in the vicinity of the Z line. If, en, as also indicated by microscopic obrvation (Peachey and Huxley, '60), the ganization of the sarcoplasmic reticulum ffers in the two types of muscles, and if, suggested by present and earlier findgs, the availability of calcium to the rcoplasm and contractile mechanism is verned by the calcium in this reticulum ianchi and Shanes, '59; Shanes and anchi, '60), it follows that transfer of ^{45} in normal Ringer's solution and under nditions of elevated K^+ and Ca^{++} may mediately involve both A and I bands slow fibers and only I bands in fast ers. To what extent this therefore inences the initial passive distribution of vated calcium in the fibers, which may scure changes in flux with activity and oduce the different effects of elevated lcium on contraction in the two systems, mains to be seen. It may also have some aring on the greater influence of calcium the depolarizability of the rectus fibers potassium noted by Fleckenstein et al. 50); indeed, the direct correlation beeen membrane potential and contracture scribed by Fleckenstein et al. might be terpreted as indicating that depolarizan supplements the effects of calcium try in contracture. In any case, these rious considerations could underlie our servation that the smaller increment in lcium influx *relative to the influx at low tassium levels* determines the smaller tassium contracture in elevated $[Ca]_o$. repetition of the observations of Peachey d Huxley from the standpoint of the ect of elevated $[Ca]_o$ would probably be lightening.

The evaluation of the correct influx m the data at hand requires correction r the limited filling of the extracellular ace and for loss of activity from the firs during washout. Since the exposure Ca^{45} was for about the half-time of filling of the extracellular space, the fibers will have taken up only ¼ the activity they would have had they been exposed instantaneously to maximum specific activity. If the half-time of washout of 91 minutes applies to the brief exposure to Ca^{45}, one should multiply the residual activity by a factor of 2.5 to obtain the fiber activity prior to washout. These factors indicate, then, that the figures in table 1 must be increased about ten-fold. (Actually, the situation is more complex because of the unknown distribution of fiber sizes. A consideration of this is beyond the scope of this paper.)

If we consider the correction factor of 10, then, and take 400 cm² as the surface area/gm, we obtain from the mean influx at 1.6 mM K^+ in table 1 0.13 μμmol/cm² sec.—somewhat larger than the estimate of 0.1 for outflux. It was pointed out in a previous analysis of radioisotope washout (Shanes and Berman, '55) that the zero time intercept is overestimated by 10% when, as in the present instance, the slow half-time is 20 times the fast half-time; this reduces the estimated influx to 0.12 μμmol/cm² sec. Consequently, within the uncertainties inherent in the outflux and influx estimates, the two unidirectional fluxes appear to be equal.

In summary, the quantitative relation between calcium influx and contraction in skeletal and heart muscle is supplemented by the temporal correlation found between potassium contracture and calcium influx in fast (twitch) and slow (tonic) striated muscle. If, as implied by these findings, the rate of calcium entry indeed controls contraction, then the increased outflux occurring under the same conditions must, as previously suggested (Bianchi and Shanes, '59; Shanes, '58), be visualized as occurring separated in space and/or time from the influx.

SUMMARY

Calcium influx in the rectus abdominis muscle of *Rana pipiens*, measured from the rate of Ca^{45} entry from Ringer's solution, is about that in the sartorius muscle, viz., 0.1 μμmol/cm² sec. Upon treatment of the muscles with 32 mM K^+, calcium influx rises by 10-fold transitorily, then remains elevated at 4-fold the resting level. As is now well-known for "slow" or "tonic"

fibers, which partly compose the frog rectus abdominis, such potassium treatment also leads to a sustained potassium contracture. Trebling of the extracellular calcium concentration trebles the passive influx of calcium, but reduces the increment of the influx in 32 mM K^+ relative to that at normal potassium concentrations; this is associated with a decreased and slower potassium contracture. K-strophanthin and thiocyanate lower the threshold potassium concentration required for contracture, by virtue of which a given elevation in K^+ causes a stronger contracture with a shorter latent period; the cardiac glycoside also increases the elevation of calcium influx induced by an increase in extracellular potassium. These findings supplement previous research on sartorius muscles of the frog that demonstrated the temporal and quantitative correlations between contraction and calcium influx to be expected from a dependence of contractile activity on the level of ionized calcium within the fibers.

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The Effect of Alloxan Diabetes on the Blood Picture of the Hamster[1]

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In the past 34 years, numerous reports have appeared in the literature indicating that changes in many blood constituents occur among patients suffering from diabetes mellitus. These changes include: leucocytosis with neutrophilia (Allan, '27; Heck, '39; Tullis, '48); depressed serum albumin levels (Lewis et al., '44; Scheurlen, '55; Schertenleib and Tuller, '58); sharply elevated alpha-2 globulin (Scheurlen, '55; Schertenleib and Tuller, '58); increased beta globulin (Lewis et al., '27; Scheurlen, '55; Schertenleib and Tuller, '58); decreased gamma globulin (Scheurlen, '55) and a rise in cholesterol and phospholipids (Schertenleib and Tuller, '58; Lever, '51; Barach and Lowy, '52; Lowy and Barach, '57) accompanied by an elevated beta lipoprotein (Lever, '51). In several instances, the degree of vascular change seemed to be in direct proportion to the severity of the case (Allan, '27; Lewis et al., '44; Scheurlen, '55). Two groups of investigators have suggested that most of the observed alterations occur either in the early years of the disease (Barach and Lowy, '52) or in patients less than 45 years old at the time of onset (Ralli et al., '57).

In the field of experimental diabetes, changes in the blood picture have likewise been noted. Pancreatectomized dogs exhibit: an elevated white cell count together with lowered red cell and hemoglobin values (Smith, '46), as well as a rise in total cholesterol which is associated with an overall increase in the serum lipoproteins, the latter being especially marked in the beta zone (Fasoli et al., '54).

Alterations in the blood picture of animals rendered diabetic through the use of alloxan are more difficult to evaluate since the possibility exists that such alterations may be due either to the direct action of this chemical upon the peripheral blood or upon the hemopoietic organs. Gualandi and Campana ('48) reported an anemia in alloxan-treated rabbits as a result of the actual destruction of circulating red blood cells coupled with damage to the bone marrow. Rose and Gyorgy ('50) showed that hemolysis of red cells occurs in rats within 15 minutes following the injection of alloxan. Mellinghoff and Zeigler ('54), using doses of from 50 to 300 mg/kg of alloxan on rabbits also observed hemolysis, recorded a great drop in red cells with a concomitant decline in hemoglobin and reported a rise in the total white cell count. The changes observed varied in proportion to the size of the alloxan dose. The time necessary for the appearance of maximum change ranged from a few hours to 11 days. These authors believed that their results were not related to diabetes. Baiardi ('55) observed the effects of diabetogenic doses of alloxan on the blood of the mouse for a period of 7 days. During that time he noted a transitory decline in the red cell count without leucocytosis but he did observe neutrophilia. In each case, the typical degenerative change in the islets of Langerhans was seen. Apparently, no attempt was made to relate any of the vascular changes to the severity of the diabetes.

In the light of these findings, it is apparent that certain questions remain unanswered: whether experimentally-induced alloxan diabetes produces the same changes in the blood seen in human diabetes mellitus; whether such changes are actually related to the diabetic state or

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are solely due to the primary action of alloxan on the blood or blood-forming organs; whether there is any relationship between blood alterations and the severity of the diabetes; and, whether the changes are common and to the same degree in all age groups.

METHODS

Over 500 male hamsters were used in this experiment. They were divided into three age groups as follows: (1) animals 4 to 5 weeks old; (2) animals 6 months old; (3) animals one year old. All hamsters, except the controls, were injected intravenously with Eastman alloxan according to the dose rate (Nace et al., '56) and technique (Pansky et al., '61) previously described. In most cases, blood studies were made at weekly intervals using the same methods reported earlier (House et al., '61). Since only those animals showing hyperglycemia one month after alloxan can be considered persistent diabetic (House and Tassoni, '57), data from such animals alone have been included here. The degree of the diabetic state was classifield as recorded elsewhere (House et al., '61).

OBSERVATIONS

Animals 4 to 5 weeks old (table 1)

It is evident that there is no leucocytosis in any of the diabetic groups. Indeed, the high diabetics actually showed a marked reduction in total white cells 12 to 14 days post-alloxan, the mean being 4460 at that time. On the other hand, there is a persistent neutrophilia with a concomitant decrease in lymphocytes among the high diabetics. This change in the white cell picture was also observed in the moderate diabetic group as late as 21 days after alloxan (30.6% polys and 64.7% lymphs) but the normal status was found by the end of one month.[3]

Although a transitory decrease in hemoglobin values is evident for about two weeks after the injection of alloxan, among the high diabetics, no significant changes were noted in the hematocrit values at any time.

A significant rise was noted in the plasma albumin of the high diabetics in the second (61.8%) and third (56.7%) weeks post alloxan. This was accompanied by proportionate reductions in the alpha and beta globulins. However, these values together with the A/G ratios, returned to normal at the end of a month.

A few individual animals showed a rise in the alpha-2 globulin during the first two or three weeks after alloxan but there was essentially no difference in this fraction after 4 weeks in any of the diabetic groups.

When the lipoproteins were considered, a significant decline in the albumin lipoprotein fraction was noted among the high diabetics at the end of one month. This same group also showed a significant fall (to 21.3%) in the alpha lipoprotein after one week. The moderate diabetics also showed a slight decline (to 36.7%) during the same period but all groups had normal alpha lipoprotein values by the end of 4 weeks. The beta-gamma lipoprotein was greatly elevated (to 60.7%) among the high diabetics one week post-alloxan and remained significantly higher at the end of one month. Less marked increases were also noted in the moderate (30.9%) and low (29.9%) diabetics during the first week after alloxan but these had returned to normal at the end of a month.

Animals 6 months old (table 2)

The white cell counts at this age level showed little deviation from the normal at the end of a month although a drop in white cells to 6350 was observed one week after alloxan among the high diabetics. On the other hand, there was a definite trend toward neutrophilia in all diabetic groups, a trend which approached statistical significance by the end of the 4th week after alloxan in the high diabetics. A simultaneous decline in lymphocytes of the same magnitude was also noted.[3]

The high diabetics showed a significant fall in hematocrit values 4 weeks after alloxan.

Since the number of animals at this age, whose blood was examined electrophoreti-

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[3] It should be noted that there is such an enormous range in percentages of polymorphonuclear leucocytes and lymphocytes, even among normal, untreated animals, that it is not possible to report these changes as statistically significant. However, the trend is very definite and a shift in range is also apparent in each case.

TABLE 1

Animals 8 to 9 weeks old

Item[1]	4 wks.—untreated			4 wks.—low diab.			4 wks.—mod. diab.			4 wks.—high diab.		
	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.
W.B.C.	4.20–13.00	7.62	2.39	3.90–10.10	6.68	2.96	4.90– 7.65	5.84	1.10	3.60–14.85	8.46	2.60
Poly.	3.0 –41.0	21.9	9.4	6.0 –47.0	22.7	11.7	11.0 –33.0	19.3	8.6	6.0 –64.0	31.4	20.0
Lymph.	56.0 –95.0	75.5	9.9	53.0 –93.0	74.7	11.8	65.0 –87.0	77.2	6.1	31.0 –91.0	67.6	18.6
Crit.	50.0 –57.0	53.5	2.3	51.0 –57.0	53.6	1.8	50.0 –52.0	50.8	0.8	44.0 –54.0	50.1	5.6
Hb.	15.8 –19.0	16.8	0.9	13.6 –18.2	16.8	0.4	14.8 –17.0	15.9	0.4	14.2 –18.2	17.1	1.1
Alb.	39.2 –58.8	48.2	5.3	38.1 –61.1	47.4	6.6	41.7 –56.1	48.9	7.2	32.3 –60.4	48.3	14.1
Alpha 1	5.7 –11.7	8.4	1.9	5.4 –15.5	9.9	3.3	6.8 –11.1	8.9	2.1	4.6 – 5.4	5.1	0.4
Alpha 2	8.2 –32.7	20.3	7.5	9.1 –33.8	20.7	9.6	14.4 –28.5	21.5	7.1	16.1 –23.7	20.7	3.2
Beta	7.6 –26.0	11.9	4.6	8.0 –25.0	12.3	5.6	11.1 –16.7	13.9	2.8	8.7 –19.4	13.5	4.4
Gam.-fib.	3.5 –19.4	11.2	3.4	7.1 –18.4	11.5	3.4	6.1 – 7.6	6.9	1.1	8.5 –19.4	12.4	4.9
A/G		0.93			0.90			0.96			0.93	
Alb. Lip.	36.0 –51.9	44.7	5.7	18.1 –52.8	36.6	9.1	45.4 –53.7	49.9	3.5	9.7 –35.1	20.7	10.0
Alpha Lip.	31.7 –44.4	39.4	5.3	23.8 –54.9	38.4	9.1	27.5 –38.0	33.9	3.4	21.3 –45.1	34.4	8.6
Bet. Gam. Lip.	11.2 –20.8	15.9	3.2	9.2 –35.6	23.9	7.6	9.2 –25.0	16.3	6.2	31.2 –65.6	44.9	13.0

[1] W.B.C., total white cell count, expressed in thousands; Poly., Neutrophil count, in per cent; Lymph., lymphocyte count, in per cent; Crit., microhematocrit values, in per cent; Hb., hemoglobin, in mg; Alb., plasma albumin; Alpha 1, alpha 1 globulin; Alpha 2, alpha 2 globulin; Beta, beta globulin; Gam-fib., gamma globulin plus fibrinogen; A/G, albumin-globulin ratio; Alb. Lip., albumin lipoprotein; Alpha Lip., alpha lipoprotein; Bet. Gam. Lip., beta-gamma lipoprotein.

TABLE 2

Animals 6 months old

Item[1]	Normal			4 wks.—low diab.			4 wks.—mod. diab.			4 wks.—high diab.		
	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.
W.B.C.	4.15–13.40	8.10	2.51	4.80–10.35	7.25	2.3	5.60–10.30	7.93	1.9	5.90– 9.10	7.50	1.0
Poly.	10.0 –37.0	21.3	2.5	18.0 –29.0	22.2	3.9	22.0 –27.0	24.3	2.1	14.0 –40.0	33.6	18.3
Lymph.	61.0 –90.0	76.3	7.7	71.0 –76.0	73.6	1.9	70.0 –76.0	72.0	2.8	11.0 –85.0	62.2	21.2
Crit.	48.0 –55.0	49.9	2.5	46.0 –53.0	49.8	2.3	47.0 –50.0	49.0	1.4	42.0 –51.0	46.3	3.2
Alb.	31.9 –55.5	45.6	7.1	53.1 –64.8	58.8	5.8	36.0 –50.7	43.4	7.3	43.0 –62.6	50.9	7.9
Alpha 1	3.4 –11.2	8.0	1.7	5.5 –11.5	8.5	3.0	6.9 –10.1	8.5	1.6	5.3 –10.1	8.0	1.7
Alpha 2	11.8 –42.2	24.7	8.6	10.4 –15.9	13.2	2.7	10.0 –16.9	13.5	3.4	12.1 –32.7	21.2	8.8
Beta	8.2 –21.4	13.1	3.8	4.8 –11.5	8.2	3.3	8.5 –13.5	11.0	2.5	9.2 –12.1	10.5	1.0
Gam.-Lib	0.0 –14.8	8.5	4.2	9.0 –13.5	11.3	2.2	23.5 –23.9	23.7	0.2	4.4 –13.3	9.5	3.8
A/G		0.84			1.43			0.77			1.03	
Alb. Lip.	28.8 –44.6	36.6	4.5	26.7 –46.1	39.3	7.4	—	—	—	39.8 –44.6	42.2	2.4
Alpha Lip.	29.8 –50.0	41.8	5.5	40.4 –47.8	44.1	3.5	—	—	—	29.5 –36.9	33.2	3.7
Bet. Gam. Lip.	10.8 –35.1	21.5	6.1	11.1 –25.6	16.8	5.5	—	—	—	23.3 –25.8	24.6	1.2

[1] Symbols used same as those in table 1.

cally was too small in any one diabetic group, no attempt was made to determine statistical significance for these values. However, certain definite trends can be reported. There was a distinct, though not large, increase in serum albumin. The alpha-1 globulin remained practically unchanged for all diabetic groups. Both the low and moderate diabetics showed some decreases in the alpha-2 globulin during the month after alloxan, but even the high diabetics had returned to normal values at the end of 4 weeks. It is noteworthy that, in both the high and low diabetic groups, which consisted of the largest numbers of animals, the A/G ratios were elevated.

The albumin lipoproteins tended to rise above normal levels in all diabetic groups. The alpha lipoproteins appeared to decline in both the moderate and high diabetics. There seemed to be little change in the beta-gamma lipoprotein in any of the diabetic groups.

Animals one year old[4]

Although no significant differences in the total white cell count was seen 4 weeks after alloxan, there was a decline in these values. This change, however, was very striking in both the moderate and high diabetics one week after treatment (from the normal 7310 to 4150).

The same tendency toward neutrophilia (a rise from 28.2% to 46.0%) and lymphopenia (a fall from 68.9% to 54.0%) was also seen in this age group, especially among the high diabetics.

There was a significant fall in hematocrit values in the high diabetics group (from the normal 50.7% to 48.0%), with lesser reductions observed among the moderate diabetics.

As was the case with the 6-month-old animals, only electrophoretic trends can be reported. At this age, the high diabetics show: a rise in plasma albumin from the normal 40.6% to 48.3% ; a very slight increase (about 6%) in beta globulin with even smaller reductions (about 3%) in the other globulin components. This resulted in an elevation of the A/G ratio from the normal 0.68 for animals of this age to 0.93, at the end of one month.

There is a tendency for the albumin liproprotein to decline from 46.5% to 33.1%, the alpha lipoprotein to remain fairly stable and for the beta-gamma fraction to increase from 17.0% to 31.7%.

DISCUSSION AND CONCLUSIONS

In only a few particulars were we able to find similarities between the blood of the human diabetic and that of the alloxan-treated hamster. The most consistent of these findings were noted in the differential white cell count, where hamsters of all ages showed the same tendency toward neutrophilia and lymphopenia. Since we did not encounter leucocytosis among our animals, it seems possible that this phenomenon is related to acidosis, for all of the human patients exhibiting high white cell counts were severely acidotic (Allan, '27; Heck, '39; Tullis, '48), a condition we were never able to demonstrate in the hamster. Neutrophilia, without leucocytosis, was also noted by Baiardi ('55) where again no acidosis was reported. The fact that leucocytosis is encountered in the pancreatectomized dog should not be surprising when it is understood that ketosis often appears following surgically-created diabetes in this animal.

The most severe cases of human diabetes have shown little, if any, change in the red cell count or in hemoglobin (Allan, '27). It has, however, been established that alloxan has a direct and deleterious effect upon both circulating red cells and bone marrow (Gualandi and Campana, '48; Rose and Gyorgy, '50; Mellinghoff and Zeigler, '54; Baiardi, '55). Thus, it seems probable that even the persistent drop in hematocrit values among the older animals and the transitory decline in hemoglobin noted among our younger animals is due to the alloxan rather than to the diabetes. In light of our own experience and that of Mellinghoff and Zeigler ('54) and Baiardi ('55), it is likely that even our hamsters who still showed anemia at the end of a month might have returned to normal if followed for a longer period.

It should be recalled that we reported a decrease in the total white cell count, a transitory decline noted especially during the first week after alloxan. This we are inclined to relate to the primary effects of the drug on all hemopoietic tissue.

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[4] Since the same general trends were shown in this age group as in the others, no table was considered necessary.

Among our younger animals, there were no significant changes noted in the plasma albumin and globulins at the end of 4 weeks although a transitory rise, similar to that reported in human diabetics by Scheurlen ('55) and Schertenleib and Tuller ('58), was observed in the alpha-2 fraction.

The transitory, but significant rise in the serum albumin of the young diabetic hamster, with a trend toward increased albumin in the older groups, has no parallel in other forms. This is diametrically opposed to the findings in man. Since this type of investigation has not been extensively used in experimental diabetes, it is not yet possible to compare these results with other animal forms. If this change proves significant, it would seem to support the view that a simple, diabetogenic dose of alloxan does not damage the kidney or liver, otherwise the albumin would have diminished.

The present report indicates that the alpha-1 globulin and the gamma globulin-fibrinogen are little affected by either the alloxan or the diabetes. Although one account (Scheurlen, '55) indicated a decline in the latter fraction, it is more generally accepted that both of these fractions remain essentially normal.

Among our younger animals, two persistent and significant changes were noted in the lipoprotein fractions, namely: a decline in the fast-moving albumin lipoprotein component and, a marked elevation in the slower-moving or stationary beta-gamma fraction. This compares favorably with human diabetic blood which shows increased lipids (Schertenleib and Tuller, '58; Lever, '51; Barach and Lowy, '57; Lowy and Barach, '57) with an elevated beta lipoprotein fraction (Lever, '51).

The 6-month-old animals appear to show a different pattern in the lipoproteins. In this group, the albumin fraction is higher, the alpha level is lower and the beta-gamma fraction remains fairly constant. In this connection, it should be pointed out that there were too few diabetics in this age group from which to draw conclusions. However, the year-old animals, in which the experimental specimens were more numerous, showed the same trend as the youngest group depressed albumin lipoprotein, a rather constant alpha fraction and a markedly elevated beta-gamma fraction. This is probably due to the same causes that account for similar changes in the pancreatectomized dog (Fasoli, '54) namely: the inadequacy of factors involved in the conversion of the large, slow-moving lipoprotein molecules in the liver into smaller, more rapidly moving molecules.

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Retention of Urea by Frog and Mammalian Kidney Slices *in vitro*[1]

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The question of how urea is handled by the mammalian renal tubules is still unsettled. In some of the lower vertebrates there is clear evidence for active tubular transport of urea. In the elasmobranch, urea is actively reabsorbed by the renal tubules, as shown by the fact that the concentration of urea in the urine is about 250 to 300 mM lower than in the plasma (Kempton, '53). In the frog, active secretion of urea has been clearly demonstrated by the finding that the amount of urea excreted in the urine is about 8 to 10 times greater than the amount filtered (Marshall, '32; Schmidt-Nielsen and Forster, '54; Forster, '54). Furthermore, Love and Lifson ('58) have shown that the flux of urea across the proximal tubule in the frog is greater in the direction from plasma to lumen than in the opposite direction.

In the mammalian kidney, the urea excretion has been found to be regulated on the tubular level. Thus, the urea clearance increases relative to the filtration rate when the nitrogen intake is increased. In rodents there is highly suggestive but not conclusive evidence that the urea clearance can exceed the filtration rate when the animals are maintained on a high protein diet (Schmidt-Nielsen, '58). When the protein intake is severely restricted, the urea clearance decreases markedly relative to the filtration rate (Schmidt-Nielsen, '55; Murdaugh, Schmidt-Nielsen, Doyle and O'Dell, '58). This latter phenomenon is particularly pronounced in ruminants (Schmidt-Nielsen, Schmidt-Nielsen, Houpt and Jarnum, '57; Schmidt-Nielsen and Osaki, '58; Schmidt-Nielsen, Osaki, Murdaugh and O'Dell, '58).

The findings suggest that the mammalian renal tubules also may be capable of active urea transport. It has been found repeatedly that substances, actively secreted by the renal tubules such as phenol red (Forster, '48) and p-amino hippurate (Cross and Taggart, '50), will accumulate in kidney slices when incubated with the substance in an oxygenated medium. With respect to urea, it has been shown by Conway and Kane ('35) that it will accumulate *in vitro* in isolated whole frog kidneys. However, in experiments with mammalian kidney slices, Conway and Fitzgerald ('42) obtained indicative but somewhat inconclusive results with respect to the ability of the slices to concentrate urea against a concentration gradient. Their results will be further dealt with in the discussion.

Since the renal tubules of frogs have been found to secrete urea actively *in vivo*, and the whole frog kidney *in vitro* was capable of accumulating urea, our initial studies were carried out with frog kidney slices to determine if they would accumulate urea from a urea-containing medium. Further studies were carried out using rat kidney slices. The results showed that both frog and rat kidney slices were capable of maintaining a urea concentration that was higher than that of the medium. Experiments with C^{14}-labeled urea showed that the excess urea found in the kidney slices must be rather firmly bound in the tissue, since it was not readily exchangeable with the labeled urea.

METHODS

Material

Kidneys of frogs and white rats were used. The frogs were *Rana pipiens* (weight

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80–150 gm) and *Rana catesbiana* (weight 230–500 gm). Prior to use, the frogs were kept in a moist tank for not more than three or four days.

The rats were adult females of the Osborn-Mendel strain weighing from 250 to 500 gm. To insure approximately the same low rate of urine flow in each animal, the rats were deprived of water for 24 hours before the experiment.

Procedure

The animals were killed and the kidneys and a piece of leg muscle, (used as control) were immediately removed and placed in iced physiological saline solutions. For frogs, this solution was made up of 100 mM NaCl, 2.5 KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.5 mM $NaH_2PO_4H_2O$ and 5.0 mM $NaHCO_3$. For mammals, it contained 144 mM NaCl, 5.75 mM KCl, 1.44 mM KH_2PO_4, 1.44 mM $MgSO_4 \cdot 7\ H_2O$ and 48 mM $NaHCO_3$ (Taggart and Forster, '52).

Tissues slices of less than 0.5 mm thickness, weighing 20–50 mg were prepared in a Stadie-Riggs microtome. In *Rana catesbiana*, the kidney was divided into two regions, proximal and distal (fig. 1). In rats, the kidney was divided into three regions: cortex, outer zone of medulla and inner zone of medulla (fig. 2).

The slices were placed in beakers containing 10 ml of the appropriate saline medium to which 0.5% glucose was added just before incubation. The beakers were placed in a Dubnoff shaker with a constant flow of 95% O_2–5% CO_2 at 37°C. In order to wash out most of the urea present in the kidney when removed from the animal, the slices were incubated for one hour in a urea-free medium. At the end of the hour, some slices were taken out for analysis and the remainder transferred to beakers containing the appropriate saline medium to which known concentrations of urea had been added. After another two hours of incubation, the slices were removed for analysis. They were blotted on filter paper, weighed on a Roller-Smith precision balance to 0.5 mg and ground to a fine homogenate in glass mortars containing 2 ml of distilled water and sand. The homogenates were centrifuged and the supernate and the incubation medium analyzed for urea and ammonia (Conway, '47). The urea concentrations in the slices were expressed in mM per liter of tissue water. The percentage water in the tissue was determined in kidney slices that had been incubated for the usual length of time. The slices were blotted on filter paper, weighed and dried to constant weight at 105°C.

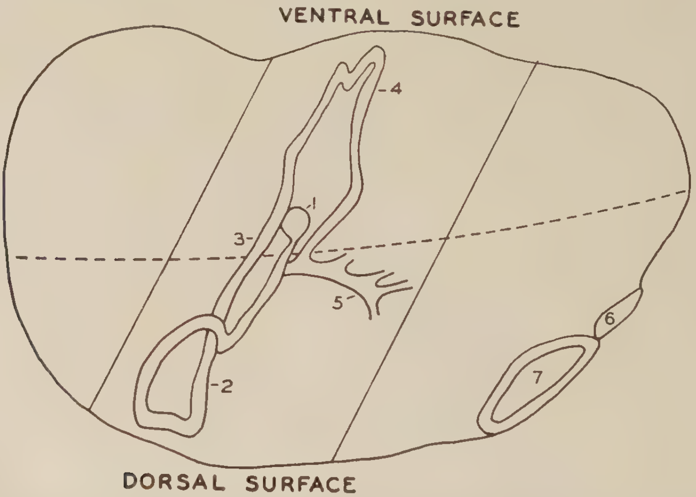

Fig. 1 Cross section of the frog kidney. Detailed portion, showing a single tubule, gives the relationships of structure of the tubule to the regions of the kidney. 1, glomerulus; 2, proximal convolutions; 3, tubular neck; 4, distal convolutions; 5, collecting duct; 6, ureter. The dotted line represents the plane of section for the preparation of the slices.

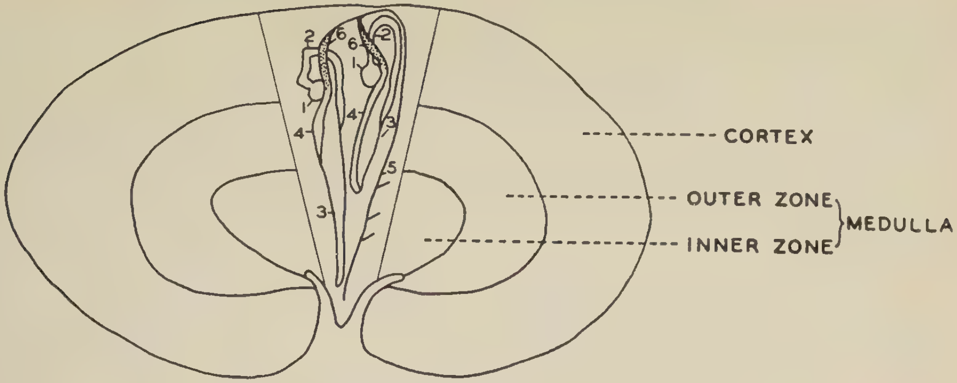

Fig. 2 Cross section of the rat kidney. Detailed portion, showing two tubules, gives the relationships of structure of the tubules to the regions of the kidney. 1, glomerulus; 2, proximal convolutions; 3, thin segment; 4, thick segment; 5, collecting duct; 6, distal convolutions. Slices were made parallel to cross section.

Metabolic inhibitors

In a series of experiments, the effect of lack of oxygen and various metabolic inhibitors was tested. The inhibitors were introduced into the incubation vessels with the urea-containing medium after the initial incubation of one hour in the urea-free medium.

The following techniques were adopted in the various experiments: (a) Pure nitrogen gas was introduced into the Dubnoff shaker at the same rate as the oxygen-carbon dioxide mixture was given to the controls.[4] (b) Cyanide concentrations were maintained throughout the two hours incubation by using a modified erlenmeyer, comparable to a Warburg vessel, equipped with a center well. A 5 M KCN solution made up in 0.1 M NaOH, was placed in the center well and served to maintain the concentration of KCN at 0.02 M[5] as well as absorb the CO_2 produced (van Heyninger, '35; Riggs, '45). (c) 2,4 dinitro-phenol was added directly to the incubation medium giving a final concentration of 0.0027 M.

Tracer studies

Studies on frog and rat kidneys were conducted using C^{14}-labeled urea. The tracer was added to the urea-containing medium in amounts calculated to give counts of about 300–1000 counts/min. in each planchet. Slices were treated as in the preceding experiments, i.e., blotted, weighed and homogenized in 2 ml H_2O. Aliquots of 100 µl of the supernate from the homogenates and samples of the medium were plated on planchets and counted using a Model D 47 (Nuclear-Chicago) gas flow counter with ultra-thin window. Aliquots of the same homogenates were analyzed chemically for urea using the Conway micro-diffusion method.

RESULTS

When frog or rat kidney slices were incubated in urea-free or urea-containing medium, they maintained at all times a higher urea concentration in the tissue water than in the surrounding medium (table 1). In muscle slices, on the other hand, the urea concentration was not significantly different from that of the incubating medium.

The amount of urea retained (i.e., the slice-medium concentration difference) by the various types of kidney slices was rather constant and characteristic for each type. In *Rana catesbiana* the dorsal portion of the kidney which contains the proximal tubules showed a greater slice-medium difference, 12.6 ± 0.60 mM/l than the ventral portion containing the distal tubules, 6.55 ± 0.91 mM/l. The difference is highly significant. The *Rana*

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[4] The medium pH was not appreciably altered by the pure nitrogen as compared with the O_2–CO_2 mixture. The pure nitrogen had no effect on the pH of the medium.

[5] The concentration of 0.02 M cyanide was used by Deyrup and Ussing ('55).

TABLE 1

Urea accumulation in tissue slices

	Number of determin.	Medium conc.	Slice-Medium conc. diff.	Treatment of slices
		mM	*mM*	
		Rana catesbiana		
Kidney				
Dorsal portion	8	0.1	12.9 ± 1.99	1 hour in urea-free medium
Ventral portion	9	0.1	5.5 ± 0.90	
Dorsal portion	16	1.69–22.7	12.6 ± 0.66	1 hour in urea-free medium followed by 2 hours in urea-containing medium
Ventral portion	9	1.69–22.7	6.55 ± 0.91	
Muscle	2	4.10–7.55	0.93	
		Rana pipiens		
Kidney	4	0.1	8.9 ± 1.1	1 hour in urea-free medium
Kidney	4	8.85–18.8	10.5 ± 1.21	1 hour in urea-free followed by 2 hours in urea-containing medium
Muscle	2	8.85–18.8	1.05 ± 1.00	
		Rattus norvegicus		
Kidney				
Cortex	7	0.1	2.94 ± 0.14	1 hour in urea-free
Cortex	56	1.69–11.3	2.79 ± 0.15	1 hour in urea-free medium followed by 2 hours in urea-containing medium
Cortex	10	2.02–14.6	2.92 ± 0.45	
Outer zone	10	2.02–14.6	3.17 ± 0.32	
Inner zone	10	2.02–14.6	1.48 ± 0.49	
Muscle	7	1.79–6.19	0.09 ± 0.36	

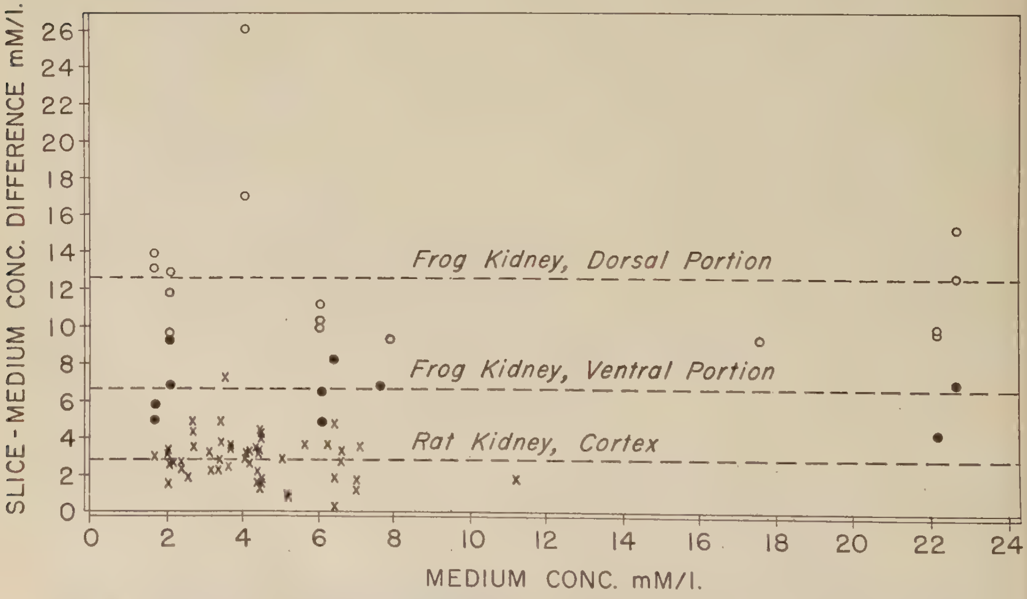

Fig. 3 Relation of slice-medium differences obtained to the medium concentration of urea in both frogs and rats. The dotted lines represent the average slice-medium difference obtained in the groups studied.

pipiens kidney slices which were not divided into dorsal and ventral portions showed an intermediary slice-medium difference of 10.5 ± 1.21 mM/l.

The rat kidney slices had a statistically significant slice-medium concentration difference of 2.94 ± 0.14 mM/l, but the difference was considerably lower than in the frog kidney slices. When the kidney was divided into zones, it was seen that while the cortex and outer zone had about the same slice-medium concentration difference, the inner zone had lower uptake.

It appears that the medium urea concentration does not influence the slice-medium concentration difference in frog or rat kidney slices. This can be seen from the fact that the difference was the same in urea-free and in urea containing media (table 1) and from the results obtained over a considerable range of urea concentrations in the incubating medium (fig. 3).

Effect of anoxia and inhibitors

If the accumulation of urea in kidney slices was due to active urea transport, it should require energy to operate. Consequently, lack of oxygen or metabolic inhibitors could be expected to decrease the difference between the concentration of urea in slice and medium.

Rana pipiens kidney slices that were incubated in an atmosphere of pure nitrogen showed a lower difference between slice and medium urea concentration than slices incubated in the oxygen-CO_2 mixture (table 2). The percentage lowering in concentration as compared with slices incubated at the same time in oxygen-CO_2 averaged 48% (range 9.5–100%).

Rat kidney slices incubated in a nitrogen atmosphere showed a definitely lower concentration than those in the oxygen-CO_2 mixture. The percentage inhibition varied from 17.0 to 100% with an average inhibition of 70.6%. This inhibition is statistically significant.

The effect of *cyanide* was studied in rat kidney slices only. The slices incubated in 0.02 M cyanide medium had a lower urea concentration than those that were in a cyanide-free medium. The percentage inhibition with cyanide ranged from 25.4% to 100.0% with an average inhibition of 70.4% (table 2). The inhibition is statistically significant.

In the presence of *2,4 dinitrophenol*, *Rana pipiens* kidney slices showed a lower slice-medium urea concentration difference than in slices without the inhibitor. The percentage lowering ranged from 49.8 to 63.1% with an average of 57.2%.

In rat kidney slices, however, no significant effect of 2,4 dinitrophenol was found.

Exchange of C^{14}-labeled urea

The preceding results showed that both frog and rat kidney slices can maintain a urea concentration that is higher than that of the incubating media and that the slice-medium concentration difference is rather constant and independent of wide variations in the medium concentration. Furthermore, it was shown that the difference was lowered when the metabolic functions of the kidney slices were interfered with.

The constancy of the slice-medium concentration difference suggested a chemical binding of urea in the tissue. In

TABLE 2

Effect of anoxia and metabolic inhibitors

	Number of determin.	Slice-medium conc. diff.		Inhibition
		Inhibited	Control	
		mM	*mM*	%
Rana pipiens				
O_2-free	6	5.15 ± 1	9.11 ± 1.04	48
2, 4 DNP	5	4.29 ± 0.7	9.69 ± 1.2	57.2
Rat				
O_2-free	10	0.78 ± 0.28	2.32 ± 0.26	70.6
Cyanide	9	0.55 ± 0.32	2.87 ± 0.24	70.4
2, 4 DNP	6	3.23 ± 0.5	3.40 ± 0.6	7.0

TABLE 3

	Chemical analysis			C^{14}
	Number of determin.	Slice-Med. conc. diff.	Slice conc. / Med. conc.	Slice counts/µl/min. / Med. counts/µl/min.
		mM/l		
Rana pipiens				
Whole kidney	8	5.63 ± 0.10	1.39 ± 0.02	0.92 ± 0.2
Rat				
Kidney cortex	6	3.92 ± 1.1	1.96 ± 0.07	0.99 ± 0.1
Kidney medulla	3	6.19 ± 1.2	1.98 ± 0.15	1.26 ± 0.2

order to investigate this possibility the exchange of C^{14}-labeled urea between medium and slice was investigated.

In the frog kidneys incubated with labeled urea, the concentration of C^{14} in the tissue water of the slice was the same as in the medium. At the same time, however, the chemical analysis showed a definite increase in urea concentration in the kidney tissue over the medium (table 3).

Comparable results were found with rat kidney slices (table 3). The labeled urea was found in the same concentration in slice and medium while the chemical concentration was greater in the slice than in the medium.

DISCUSSION

Both frog and rat kidney slices were able to maintain a concentration of urea above that of the incubating medium. This fact was borne out in all experiments conducted. Although the degree of concentration varied with species and the anatomical part of the kidney, the general pattern was the same. In all kidney slices the concentration difference between slice and medium appeared to be constant and independent of the medium urea concentration.[6]

From the chemical analysis, it would appear that urea was taken up from the medium by the kidney slices. However, the results of the isotope experiment showed that in these animals[7] urea was not actively taken up from the medium since the concentration of labeled urea was the same in the tissue water as in the medium. This would indicate that the difference in chemical concentration of urea found in slice and medium is due to chemical binding of urea. This binding must be very firm since the urea failed to exchange with the isotope in the medium during two hours of incubation. On the other hand, the binding appeared to be associated with normal metabolic processes of the tissue since anoxia or metabolic inhibitors released part of the bound urea.

The binding of urea appears to be characteristic of the renal tissue since the muscle tissue did not show any such binding. The question now is whether or not the ability of the kidney to bind urea can be related to the physiological handling of urea by the renal tubules.

In the frog kidney, the largest amount of bound urea was found in the dorsal portion containing the proximal tubules. The active urea transport presumably is located primarily in the proximal tubules of the frog (Walker and Hudson, '37). In the rat kidney the binding was most pronounced in the cortex and outer zone of the medulla. This of course does not indicate whether the binding is more pronounced in proximal or in distal convolutions, but it does suggest that there is little binding in the thin limbs of the loops of Henle. In micropuncture studies it has been found that at the end of the proximal convolutions only ⅓ of the filtered urea is still present within the tubule, while in the distal convolutions the amount of urea present is equal to or exceeds the amount filtered (Lassiter, Gottschalk and Mylle, '60; Schmidt-Nielsen, Ullrich,

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[6] A similar retention of urea *in vivo* in the frog kidney was found by Marshall and Crane ('24) and by Forster ('54). The urea concentration in the kidney exceeded the concentration in the blood by 2–12 mM/l showing that the order of magnitude of the retention *in vivo* is about the same as that found *in vitro*.

[7] Hamster and sheep kidney slices have shown active uptake of C^{14}-labeled urea from the medium (in preparation).

O'Dell, Pehling, Gottschalk, Lassiter and Mylle, '60). If urea is actively secreted by the tubules in the rat it is therefore most likely that it occurs along the distal tubule.

Substances that are actively transported by the kidney are often found to accumulate when slices of the tissue are incubated *in vitro* with the substance. From many different investigations it appears that in the passage of the substance across the renal epithelium it forms a complex with a carrier molecule (Shannon, '39; Beyer, '50; Deyrup and Ussing, '55). Foulkes and Miller ('59) found that the accumulated PAH in kidney slices consisted of two components, one fraction which rapidly equilibrates with the medium, and a second fraction which diffuses and equilibrates slowly and is responsible for the high slice to medium concentration ratio. Apparently the slowly equilibrating fraction represents the bound PAH. The binding of PAH was found to be sensitive to metabolic inhibitors, which caused the complex to break down and accelerated the leakage of PAH from the cells. Chinard ('56) found that the excretion of actively secreted PAH was delayed relative to the filtered fraction and calculated that the average time it takes for PAH to cross the tubular epithelium is as much as 90 seconds. He deduced that PAH is bound to a carrier molecule of large molecular size, and that the time required for the transport is related to the time it takes for the complex to diffuse from one side of the cell to the other. Copenhaver and Forster ('58) found that the accumulated PAH in kidney slices can be displaced by other substances which apparently have a greater affinity for the carrier molecule. The competitive substances that displace PAH are usually less actively transported than PAH.

One can draw an analogy between our findings with urea and the findings with PAH. Since urea was present in the kidneys when excised from the animals, it could be assumed that the carrier was already saturated with urea when the slices were prepared. While the urea which was not bound in the tissue would diffuse out into the urea free medium, the bound urea would remain on the carrier which then could not bind urea from the medium when the slices were placed in urea. This suggestion is to a certain degree substantiated by the findings of Conway and Kane ('35) with frog kidneys and by Conway and Fitzgerald ('42) with rat kidney slices. They found that kidneys in which all bound urea had been removed by perfusion with a cyanide solution would take up urea from a urea-containing cyanide-free medium. In experiments with whole frog kidneys, they found an average urea slice-medium difference of 4.2 mM. The experiments with rat kidney slices strongly indicated an uptake of urea, but were not as conclusive as the frog data, because the investigators used a relatively high medium urea concentration (33 mM). Their data are given as percentage uptake by the slice and it can be calculated from their results that the slice-medium concentration difference was around 2–3 mM. The order of magnitude is the same as in our experiments, and the findings indicate that if urea has been removed from the carrier system it can be taken up again from the medium.

In both the PAH system and the urea system the binding was found to be sensitive to metabolic inhibitors. It is interesting to note that the frog and rat kidney slices responded differently to 2,4 dinitrophenol. In the frog kidney slices, the bound urea was released by 2,4 dinitrophenol but this was not the case in the rat kidney slices. The results are consistent with *in vivo* findings with this inhibitor. Forster ('54) found inhibition of active secretion of urea by 2,4 dinitrophenol in normal frogs. No effect of the drug has been found on the urea excretion in rats, even under conditions where the animals had been maintained on a high protein diet for a period of time and the urea clearance exceeded the true endogenous creatinine clearance by as much as 40% (B. Schmidt-Nielsen, unpublished).

The main difference between the binding of urea in the kidney slices and that of PAH lies in the firmness of the binding. Foulkes and Miller ('59) found that approximately ⅓ of the bound PAH exchanged with PAH in the medium in 12 minutes. Similarly, Copenhaver and Forster ('58) found that about half of the accumulated PAH in kidney slices will run out in 30 minutes when the slice is placed in a PAH-free medium. In our experiments, no measurable exchange was found between the bound urea in the kidney slice and the labeled urea in the medium in the course of two hours.

The present findings are not conclusive, but the considerable similarities between the handling of urea by rat and frog kidney slices, as well as the similarities between the handling of PAH and urea by rat kidney slices suggest that the binding of urea by the renal tissue is associated with a urea transport system.

SUMMARY

1. Thin slices of the kidneys of rats and frogs incubated in physiological saline media consistently maintained a higher urea concentration than that of the medium.

2. In both rat and frog kidney slices the slice-medium concentration difference was independent of the medium-urea concentration.

3. The slice-medium concentration difference was higher in frog kidney slices than in the rat kidney slices. In frog kidneys the difference was greater in the dorsal portion (12.9 ± 1.99 mM/l) than in the ventral portion (5.5 ± 0.90 mM/l). In rat kidneys the difference was 2.94 ± 0.14 mM/l in the cortex, 3.17 ± 0.32 mM/l in the outer zone, and 1.48 ± 0.49 mM/l in the inner zone.

4. Anoxia and metabolic inhibitors significantly lowered the slice-medium concentration difference in both rat and frog kidney slices. 2,4 dinitrophenol lowered the slice-medium concentration difference in frog kidney slices, but had no effect on the rat kidney slices.

5. When C^{14}-labeled urea was present in the incubation medium it was found to equilibrate evenly between medium and slice. From this it appears that urea was not actively taken up from the medium but was present in the slice in some bound form.

6. A possible explanation of the binding in terms of a transport carrier system is advanced.

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